A Genetically Engineered, Protein-based Optical Biosensor of Myosin II Regulatory Light Chain Phosphorylation*

(Received for publication, September 9, 1993, and in revised form, January 28, 1994)

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Myosin II is an important motor in the contraction of smooth and striated muscle as well as in a variety of non-muscle cell motile events including cytokinesis, cortical contractions during migration of fibroblasts, and capping of receptors. Phosphorylation of the 20-kDa light chain by myosin light chain kinase is part of the regulation of smooth muscle and mammalian nonmuscle myosin II. We designed, characterized, and tested the use of a protein-based optical biosensor to monitor this phosphorylation "switch." A regulatory light chain was genetically engineered to contain a single cysteine at amino acid position 18. The mutant light chain (Cys18-LC20), reacted with the fluorophore acrylodan, responded to phosphorylation of serine 19 with a fluorescence emission quenching of 60% and a 28-nm red-shift. When the acrylodan-labeled mutant light chain (AC-Cys(18,Lc)), was exchanged into turkey gizzard myosin II, it exhibited a 25% fluorescence emission quenching and a 10-nm red-shift upon phosphorylation of serine 19. The myosin II optical biosensor exhibited nearly control levels of the rate of phosphorylation, K'ATPase activity, and in situ motility. The acrylodan-labeled light chain was exchanged into the A-bands of chicken pectoralis myofibrils in situ to demonstrate the localization and activity of the biosensor in a highly ordered contractile system. Fluorometry and quantitative fluorescence microscopic imaging experiments demonstrated that AC-Cys(18,Lc) exchanged myofibrils expressed a phosphorylation-dependent fluorescence change. Labeled light chains were also incorporated into stress fibers of living fibroblasts and smooth muscle cells. This general approach of combining molecular biology and fluorescence spectroscopy to create novel protein-based optical biosensors should provide valuable tools for investigations with model systems and solution studies and ultimately yield important information about temporal-spatial chemical and molecular changes in live cells.

The activity and conformation of both smooth muscle and mammalian nonmuscle myosin II is regulated by phosphorylation of serine 19 of the 20-kDa regulatory light chain by myosin light chain kinase (MLCK). Under physiological conditions in vitro, light chain phosphorylation transforms smooth muscle and mammalian nonmuscle myosin II (hereafter referred to as "myosin") from a folded 10S to an extended 6S conformation, increases the absorbance of the monomers, increases the actin-activated MgATPase activity, and moves actin filaments (reviewed in Refs. 1–5). Investigations of live cells (6–8) and extracted cell models (9, 10) suggest a similar role for myosin regulatory light chain phosphorylation in vivo, yet currently there is no way to follow the temporal and spatial distribution of this important event in living cells.

Dynamic chemical and molecular events may be mapped within living cells using fluorescent analog cytometry (11, 12, 13). In addition, fluorescent physiological indicators that are sensitive to their surrounding environment have been engineered to exhibit changes in fluorescence lifetime, quantum yield, excitation spectrum, or emission spectrum in response to specific chemical changes. Environmentally sensitive fluorescent indicators are now available for a wide range of parameters such as free calcium, pH, membrane potential, and proximity (14, 13).

A new class of fluorescent analog, termed "protein-based optical biosensors," combines the molecular specificity of a peptide or protein with the sensing capability of physiological fluorescent indicators (15). MeroCaM, a calmodulin-activation sensor (16, 17), and FICRBR, an indicator of cAMP signaling (18), are two such probes. Both biosensors were prepared using traditional biochemical labeling procedures. Here we report the development of a myosin regulatory light chain phosphorylation biosensor that responds spectroscopically to phosphorylation by MLCK. We have used a genetically engineered protein to place an environmentally sensitive fluorophore adjacent to the MLCK-phosphorylated serine. The biochemical properties of the myosin II analog are similar to those of controls, and the results of in situ studies with myofibrils demonstrate that the approach works. Optimization of this probe for live cell investigations is in progress.

This approach of genetically engineering a peptide or protein so that an optimally constructed, environmentally sensitive, fluorescent probe can be placed in specific regions of the molecule should guide the creation of new protein-based optical biosensors. This will ultimately elucidate the temporal-spatial dynamics of the regulatory and effecter events during a wide variety of cell and tissue functions (13).

* This research was supported by National Science Foundation Science and Technology Center Grant BIR-8920118, National Institutes of Health Grant AR32461 (to D. L. T.), and National Institutes of Health Grant HL38113 (to K. T.) The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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‡ Established Investigator of the American Heart Association.
† The abbreviations used are: MLCK, myosin light chain kinase; CaM, calmodulin; AC-Cys(18,Lc), acrylodan-labeled Cys18 mutant 20-kDa light chain; wLc20, gizzard-purified 20-kDa light chain; Cys18,Lc20, Cys18 mutant 20-kDa light chain; MOPS, 4-morpholinepropanesulfonic acid; DTT, dithiothreitol; ATPγS, adenosine 5′-O-thiotriphosphate; IAEDANS, 5-[(2-iodoacetoxy)ethyl]aminomethylamino]naphthalene-1-sulfonic acid; IANBD, N-[2-iodoacetoxyethyl]-N-methylamino-7-nitrobenz-2-oxa-1,3-diazole; BODIPY FL IA, N-[4,4-di-fluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl]-N-iodoacetylpropidylethylamidine; BODIPY 530/550, N-[4,4-di-fluoro-5,7-diphenyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl]-N-iodoacetylpropidylethylamidine; Br-BODIPY 493/503, 8-bromomethyl-4,4-di-fluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene.
Myosin II LC_{20} Phosphorylation Biosensor

**MATERIALS AND METHODS**

**Reagents**

Adenosine 5'-triphosphate, tetra(triethylammonium) salt (γ-32P) (2 mCi/mM) was purchased from New England Nuclear/DuPont and Amer sham (Amersham, Buckinghamshire, England). Guanidine hydrochloride (IGC Bi; cals, Cleveland, OH) was ultrapure. All fluorescent dyes were obtained from Molecular Probes (Eugene, OR). Glucose oxidase (Sigma) activity was 15,000–25,000 units/mg and catalase (Sigma) activity was 20,000–50,000 units/mg. All other chemicals were from Sigma and Fisher Scientific.

**Protein Purification**

*Myosin Regulatory Light Chains—Turkey gizzard myosin regulatory light chains were purified according to a procedure kindly provided by Jim Sellers. Myosin was prepared (starting with 500 g of turkey gizzards) as described (19). To extract the 20-kDa light chains, the myosin pellet was resuspended in 50 mM NaCl, 10 mM MOPS, 0.1 mM EGTA, 1 mM MgCl2, 1 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.0, at 4 °C to a concentration of about 20 mg/ml. Guanidine hydrochloride (6 M), Tris (20 mM), EDTA (2 mM), and DTT (5 mM) were added to make a volume of 100 ml, and the solution was stirred overnight at 4 °C. To precipitate the heavy chains, 1 volume of cold 100% EtOH with vigorous stirring. After 30 min of stirring, the solution was centrifuged at 13,700 x g for 30 min, then the supernatant was filtered through glass wool. Ethanol was rotary evaporated from the supernatant and the light chain solution was dialyzed against light chain buffer (10 mM MOPS, 0.1 mM EGTA, 1 mM MgCl2, 1 mM Na2S, pH 7.0 at 4 °C). Any precipitated light chains were dissolved in a small volume of 6 M urea, 10 mM MOPS, 0.1 mM EGTA, 1 mM MgCl2, and 1 mM DTT, pH 7.0 at 25 °C. Dissolved light chains were applied to a 10-ml DEAE-cellulose (DE-52, column equilibrated in the above buffer and eluted with a 1–300 mM NaCl (100 ml) gradient in the same buffer. Mixed light chains were separated on a hydroxylapatite column as described by Stafford (20). Pure LC_{20} was concentrated and stored at -20 °C until needed.

*Mutant Myosin Regulatory Light Chains—Site-directed mutagenesis of AC-Cys's.LC_{20} (Amersham Corp., Mutagenesis Kit) was used to engineer a mutant gizzard regulatory light chain with a single cysteine at residue 18. The endogenous Cys^{t-18} was replaced by an alanine (TGC). The mutant Cys^{t-18} regulatory light chain was cloned into the EcoRI site of pT7-7, expressed in E. coli strain BL21 (DE3), and purified as described (21).

*Other Proteins—Chicken pectoralis myofibrils, turkey gizzard myosin, turkey gizzard myosin light chain kinase, and bovine brain calmodulin (CaM) were purified as described (22). Phosphorylated versus time were calculated and compared with the slopes of graphs of percent myosin phosphorylated versus time were calculated and compared with the control (myosin containing wtLC_{20}).

**Fluorescence Labeling and Spectroscopy**

*Light Chain Exchanges—Regulatory light chain exchanges into smooth muscle myosin were performed as in (19) with some minor modifications. Light chains were exchanged into myofibrils as follows. Washed myofibrils (20 µl) were exchanged with acrylodan-labeled Cys^{t-18} mutant light chains (AC-Cys^{t-18}LC_{20}) (30–40 µM) at pH 7.0 (50 mM EGTA) (5 µM) in 10 mM EDTA at 37 °C for 15 min (all dilutions were made in light chain buffer). The samples were put on ice and MgCl2 was added to 12 mM. Rhodamine phalloidin (Molecular Probes) was then added to the AC-Cys^{t-18}LC_{20} exchanged myofibrils (but co-exchanged myofibrils) at a concentration of 1 unit/ml and incubated at room temperature for 10 min. The solution was diluted with the addition of 500 µl of rigor buffer and centrifuged at 3,000 x g for 10 min to remove unbound light chains and rhodamine-phallolidin if added. The supernatant was removed, and the pellet was gently resuspended in 10 µl of rigor buffer.

*Fluorescence Labeling and Spectroscopy—Light Chain Labeling—Gizzard-purified myosin 20-kDa light chains (wtLC_{20}) or Cys^{t-18} mutant light chains (Cys^{t-18} LC_{20}) were fluorescently labeled at pH 8.0 according to Marsh and Lowey (24) with the following modification. After termination of the labeling reaction, free dye was removed by repeated dilution with labeling buffer and concentration of the solution in Centricon-10 microconcentrators (Amicon; Beverly, MA). Labeled light chains were then dialyzed into light chain buffer to remove any remaining dye and finally clarified at 100,000 x g for 1.5 h. SDS-polyacrylamide gel electrophoresis of the labeled light chain demonstrated that the fluorescence appeared as a single band, which later corresponded to the Coomassie Brilliant Blue-stained light chain band with the same apparent molecular weight. The fluorescence at the gel front, indicating that no covalently bound dye was present in the labeled protein samples. AC-Cys^{t-18}LC_{20} solutions with dye-to-protein ratios of ~10 were selected for the biochemical characterization experiments. Extinction Coefficients—A precise amount of acrylodan was solubilized in dimethylformamide and reacted with a 100-fold molar excess of cysteine in 7% guanidine hydrochloride, 0.5 mM Tris, pH 8.0, at room temperature for 3 h. The absorbance spectrum of acrylodan conjugated to Cys^{t-18}LC_{20} (in the above buffer) did not differ significantly in peak shape or λmax (390 nm) from acrylodan-conjugated cysteine in this buffer. Therefore, the extinction coefficient of acrylodan reacted to cysteine was used as the extinction coefficient of acrylodan-conjugated Cys^{t-18}LC_{20}, in 7% guanidine-HCl, 9.5 mM Tris, pH 8.0. An adjustment was made to this extinction coefficient for AC-Cys^{t-18}LC_{20} in light chain buffer. The resulting extinction coefficient of acrylodan reacted to Cys^{t-18}LC_{20} in light chain buffer is 11,900 cm⁻¹-mmol⁻¹ at 360 nm. This number may be compared with the extinction coefficient of unreacted acrylodan in water reported by Prendergast et al. (25) of 12,900 cm⁻¹-mmol⁻¹ at 360 nm. The extinction coefficient used here for a 1 mg/ml wtLC_{20} or Cys^{t-18}LC_{20} solution at 280 nm is 0.30.

**Dye-to-Protein Ratios—Acrylodan concentrations were determined by measuring the absorbance of the acrylodan-reacted protein at 360 nm, using the extinction coefficient measured above, and applying Beer's Law. Light chain concentrations were determined with a BSA protein assay of acrylodan labeled light chains, using unlabeled light chains for a standard curve.

**Fluorescence Spectroscopy—**All fluorescence excitation and emission scans were recorded on a SPEX Fluorolog-2 system corrected for the intensity of the exciting light and sensitivity of the detection system.

**Biochemical Assays**

**Phosphorylation Rate Assays—**Phosphorylation rate assays were performed according to the procedure of Adelstein and Klee (26) with minor modifications in reagent concentrations. The light chain phosphorylation assays were performed in light chain buffer with the addition of 1 µM CaM, 1 mM CaCl2, 19 mM MgCl2, and 1 mM ATP (all were saturating concentrations). 1 mM MLCK, 3 mM DTT, and 13.5 µM time point of [γ-32P]ATP were also used. The assay was started with the addition of 0–15 µM native or mutant regulatory light chains. Aliquots were removed every 1 min for a period of 3 min/light chain concentration. The remainder of the assay was performed as described (26). Exchanged myosin phosphorylation assays were as above, but with 2 mM MLCK and 50 mM KCl. The assay was started with the addition of 2.5 µM myosin. Aliquots were removed every 30 s for 1.5 min. Unphosphorylated light chains were exchanged into thio-phosphorylated myosin so that the only the rate of exogenously added light chains would be measured. Percent exchange of each light chain into myosin was as follow:

- wtLC_{20}: 55%, Cys^{t-18}LC_{20}: 70%, AC-Cys^{t-18}LC_{20}: 40%. To compare phosphorylation rates of the three myosins, initial velocities were collected for each exchanged myosin, and the percent of phosphorylated myosin was calculated for each time point. The slopes of percent myosin phosphorylated versus time were calculated and compared with the control (myosin containing wtLC_{20}).

**Other Activity Measurements—**The in vitro motility assay was performed at 30 °C (Vermont Technologies hepted nicotine) as previously described in detail (19). EDTA/ATPase assays were performed in 0.5 mM KCI as published (27).

**Electrophoresis**

SDS-polyacrylamide gel electrophoresis of regulatory light chains was in 12.5% gels according to Laemmli (28). Urea/glycerol gels were prepared according to the procedure of Trybus and Lowey (29).

**Cell Culture**

Swiss 3T3 fibroblast cells (American Type Culture Collection No. CCL22; passage 118–128) were cultured, plated onto coverslips, and serum deprived as described (30). Smooth muscle cells (ATCC No. CRL14444 A76); cells from ATCC were passed 1–6 times) were cultured in Dulbecco's modified Eagle's medium without phenol red plus 10% fetal bovine serum and penicillin/streptomycin. Smooth muscle cells were plated as described (30) at a density of 3 x 10^5 and used for experiments 2 or 3 days later.

**Microinjection**

Smooth muscle and Swiss mouse 3T3 fibroblast cells were microinjected with fluorescent light chains or mutant light chains as described (31). Light chains were co-exchanged in light chain injection buffer (2 mM HEPES, 0.1 mM EGTA, 1 mM DTT, pH 7.3 at 25 °C); cells were allowed to recover for 3–12 h before imaging. The culture medium was
changed to Earles plus essential amino acids with 0.2% calf serum and penicillin/streptomycin for cells containing acrylodan to minimize autofluorescence of the medium.

Microscopy and Image Analysis
Fluorescence microscopy and image acquisition were performed on a BDS Multimode microscope (Biological Detection Systems, Pittsburgh, PA). Cellular experiments were performed in a sealed climate controlled chamber as described (6). AC-Cys\(^{18}\).LC\(_{20}\) injected cells were imaged using a 0.6 NA 40X Plan-ULTRAFLUAR objective (Carl Zeiss, Inc., Thornwood, NY), an acrylodan filter set (360 nm excitation filter with a 20-nm bandpass, 400-nm longpass dichroic, 500-nm emission filter with a 100-nm bandpass), and a 100 watt mercury arc lamp equipped with a quartz condenser. Rhodamine-Cys\(^{18}\).LC\(_{20}\)-injected cells were imaged with a 1.3 NA 100X Plan NEOFUOR objective (Zeiss), a rhodamine filter set (540-nm excitation filter with a 23-nm bandpass, 572-nm longpass dichroic, 590-nm emission filter with a 35-nm bandpass), and a 75 watt xenon arc lamp. A cooled CCD camera (Photometrics, Ltd.) was used to acquire all images.

Exchanged myofibrils (5 μl) plus 1 μl of oxygen scavengers (final concentrations: 3 mg/ml glucose, 0.7 mg/ml glucose oxidase, 0.07 mg/ml catalase) were incubated for 1.5 (2.5 cm) round quartz coverslip (Quartz Scientific, Fairport Harbor, OH), and the opposite side of this microscope chamber was covered with a no. 1.5 (22 × 50-mm) glass coverslip. The chamber was sealed with clear nailpolish. Imaging was performed as described above, but with a 1.2 NA 100X Plan-ULTRAFLUAR objective (Zeiss).

Phosphorylation-based fluorescence changes in exchanged myofibrils were detected as follows. Washed myofibrils were co-exchanged with AC-Cys\(^{18}\).LC\(_{20}\) (30 μM) and rhodamine-wtLC\(_{20}\) (5 μM). Fluorescence experiments are described in further detail under "Results." Myofibrils in rigor buffer plus oxygen scavengers (same concentrations as above) were pipetted onto a 1.5 (2.5-cm) round quartz coverslip to which two Saran Wrap\textsuperscript{®} spacers were attached to form a flow channel. An irrigation chamber was constructed by sealing the quartz coverslip with the myofibrils to a no. 1.5 (22 × 50-mm) glass coverslip on all sides but the flow channel. Acrylodan fluorescence images were collected with the multimode microscope of many unphosphorylated myofibrils for 1 s with a neutral density filter (as described above), using the rhodamine fluorescence to focus. In this manner, acrylodan photobleaching was minimized since acrylodan was only excited during the 1-s image acquisition. A phosphorylation solution (rigor buffer plus 0.4 μM MLCK, 1 μM CaM, 2 mM ATP\(_{y}\)S, 1 mM CaCl\(_2\), and bleaching inhibitors as above) or a control solution (rigor buffer plus 2 mM ATP\(_{y}\)S, 1 mM CaCl\(_2\), and bleaching inhibitors as above) were flowed into the chamber. After a 45-min incubation on the microscope stage, a second image of each phosphorylated myofibril previously imaged was collected using NIH Image (version 1.47), and fluorescence intensity changes were calculated.

RESULTS

Fluorescent Analogs of Native Regulatory Light Chains Are Insensitive Phosphorylation Indicators—The native smooth muscle myosin regulatory light chain (wtLC\(_{20}\)) contains 1 cysteine, at amino acid position 108, allowing for specificity when labeling with sulphydryl-reactive fluorescent dyes. Initially, wtLC\(_{20}\) was labeled with various environmentally sensitive and insensitive sulphydryl-reactive fluorescent dyes. The rationale was that the region around cysteine 108 may undergo a phosphorylation-dependent conformational change that would cause a change in fluorescence properties of the dye. Native light chains were labeled with the sulphydryl-reactive derivatives of acrylodan, IAEHAND, NBD-chloride, IANBD, fluorescein, and rhodamine. They were exchanged into the endogenous light chains into chicken gizzard smooth muscle myosin, where they were completely phosphorylatable by smooth muscle MLCK. These analogs did not, however, change their fluorescence properties after phosphorylation by MLCK, whether tested alone in solution or after exchange into gizzard myosin. Nevertheless, some of these native regulatory light chain analogs could be used as intracellular tracking indicators of myosin (15).

Development of a Mutant Regulatory Light Chain—The inability to develop a fluorescent indicator from wtLC\(_{20}\) encouraged us to explore site-specific mutagenesis. We decided to move the cysteine closer to the site of phosphorylation, so that the phospho group could be directly detected with a charge-sensitive fluorophore. A chicken gizzard smooth muscle regulatory light chain was genetically engineered to contain a single cysteine at position 18 immediately adjacent to the serine phosphorylated by MLCK (see Fig. 1). This mutant serves as the first of many possible phosphorylation indicators of LC\(_{20}\).

Amino acid position 18 was chosen as the initial site for the cysteine position based on previous functional assays. Kemp et al. (32) found that deletion or modification of lysine 11, lysine 12, or arginine 13 on the regulatory light chain (see Fig. 1) had a major influence on the kinetics of light chain phosphorylation and Pearson et al. (33) have demonstrated that residues on the carboxyl terminal side of serine 19 (valine 21 and phenylalanine 22) are important for proper \(V_{\text{max}}\) of phosphorylation. Ikue and Morita (34) identified the amino acid stretch from arginine 13 to arginine 16 (see Fig. 1) as being essential for proper light chain phosphorylation enhancement of myosin actin-activated MgATPase activity. We wished to avoid modifying one of the above residues in order to minimize mutational effects on light chain structure and function. Threonine 18 is obviously in close proximity to serine 19; furthermore, since it is a secondary site of phosphorylation by MLCK (35, 36), we reasoned that it was not an important MLCK "binding" residue.

Acrylodan-labeled Cys\(^{18}\).LC\(_{20}\) Responds to Phosphorylation by Changes in Spectroscopic Properties—Fluorescent Cys\(^{18}\).LC\(_{20}\) analogs (labeled with either acrylodan, BODIPY, IAEHAND, IANBD, fluorescein, or rhodamine) were examined spectroscopically for serine 19 phosphorylation-sensitivity. Light chains were phosphorylated by the addition of MLCK, CaM, CaCl\(_2\), and MgATP to the sample in the fluorometer. Excitation and emission scans were collected for phosphorylated and phosphorylated labeled light chains. Unphosphorylated AC-Cys\(^{18}\).LC\(_{20}\) control scans were used to correct for dilution of the dye and photobleaching. Urea/glycerol gel analysis of the fluorescently labeled light chains confirmed that the control samples were 100% unphosphorylated and the experimental samples were 100% phosphorylated. All fluorescently labeled Cys\(^{18}\).LC\(_{20}\) analogs, with the exception of AC-Cys\(^{18}\).LC\(_{20}\), exhibited a 0–23% fluorescence quenching and no excitation or emission wavelength shift upon phosphorylation of serine 19. AC-Cys\(^{18}\).LC\(_{20}\) displayed a 28-nm emission wavelength shift to the red region of the spectrum and a maximum of a 60% quenching shift.
fluorescence properties. Light chain phosphorylation produces a maximum acrylodan fluorescence quenching of 60% and a 28-nm red-shifted wavelength peak over control, unphosphorylated samples. Excitation (A) and emission (B) fluorescence scans were acquired of unphosphorylated (- - -) or phosphorylated (-----) AC-Cys'*.LC17 (0.25 mg/ml). Scans were collected of phosphorylated AC-Cys'*.LC20 until no further change in fluorescence occurred (usually maximal change occurred in 5 min). Light chains were 100% phosphorylated as determined by urea/glycerol gel electrophoresis.

of fluorescence intensity over control scans upon phosphorylation by MLCK. The analog also exhibited a 40% decrease in excitation intensity upon phosphorylation (Fig. 2). Similarly, Bowman and Stull (37) reported a fluorescence quenching of an acrylodan-labeled synthetic peptide upon phosphorylation by rabbit skeletal MLCK.

The phosphorylation-dependent fluorescence wavelength shift exhibited by AC-Cys'*.LC20 is a particularly useful type of fluorescence change for ratio measurements for live cell studies. This fluorescent analog was selected as the phosphorylation biosensor and was subjected to further spectroscopic and biochemical characterization.

Myosin Containing AC-Cys'*.LC20 Detects Phosphorylation by Changes in Spectroscopic Properties—AC-Cys'*.LC20 was exchanged into smooth muscle myosin to approximately 40% (Fig. 3) and then tested for spectroscopic changes upon phosphorylation as for the labeled light chains (Fig. 4). AC-Cys'*.LC20 exchanged myosin exhibited approximately a 10-nm emission fluorescence wavelength shift to the red and a maximum of a 25% fluorescence quenching in emission. The exchanged myosin also exhibited a 16% quenching of fluorescence intensity in excitation scans (Fig. 4). Urea/glycerol gel analysis showed that unphosphorylated control samples were 100% unphosphorylated, and phosphorylated samples were approximately 85% phosphorylated. The IAEcDANS, IANBD, fluorescein, and rhodamine-labeled Cys'*.LC20 analogs were also exchanged into gizzard myosin and screened for phosphorylation sensitivity. Some of these exchanged myosin analogs exhibited small fluorescence quenching, but none showed a fluorescence wavelength shift upon phosphorylation.

AC-Cys'*.LC20 Shows a Rate of Phosphorylation Similar to wtLC20—Table I compares the Kp and Vmax values for phosphorylation of free wtLC20, unlabelled Cys'*.LC20, and AC-Cys'*.LC20 alone and after exchange onto smooth muscle myosin. The initial velocities of phosphorylation of free light chains were determined as a function of light chain concentration (0–15 um). CaM, ATP, and CaCl2 were used at saturating concentrations. Table I shows unlabeled Cys'*.LC20 to be as good a substrate for MLCK as is wtLC20. The presence of acrylodan on cysteine 18 only slightly increased the Kp of phosphorylation relative to wtLC20. The Vmax of AC-Cys'*.LC20 is less than that for both wtLC20 and unlabeled Cys'*.LC20, but is still within the range of 10–30 μmol PO4/mg MLC/min reported for phosphorylation of wtLC20 by MLCK (26).

The rate of phosphorylation of bound light chains was determined by exchanging unphosphorylated light chains into thiophosphorylated myosin. Only the rate of exogenously added light chains will be measured since endogenous light chains will not participate in the assay. The rate of phosphorylation of myosin containing AC-Cys'*.LC20 is similar to that of myosin containing wtLC20 (Table I).

Activity and Regulation of Myosin Containing AC-Cys'*.LC20—Neither the mutation nor the dye on the mutant light chain was found to significantly affect the exchanged myosin K'EDTA ATPase activity (see Table II). We also employed the gliding filament motility assay (38) to determine the ability of myosin containing AC-Cys'*.LC20 to regulate actin movement and to quantitate the velocity of the actin movement. Phosphorylated light chains were exchanged into an unphosphorylated myosin so that only those myosins that received a phosphorylated light chain would be "switched on." In this way, we could directly measure the ability of AC-Cys'*.LC20 to regulate native gizzard myosin; any endogenous wtLC20 that did not exchange out of the myosin would be unphosphorylated and "silent."

Table III shows the results of the motility assay. Unphosphorylated exchanged myosins could not move actin; only phospho-
Myosin II LC\textsubscript{20} Phosphorylation Biosensor

TABLE III

<table>
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<th>Actin movement by exchanged myosins</th>
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| Phosphorylated wtLC\textsubscript{20}, Cys\textsuperscript{18}-LC\textsubscript{20}, or AC-Cys\textsuperscript{18}-LC\textsubscript{20} were exchanged into unphosphorylated smooth muscle myosin II. The rates at which these exchanged myosin move single actin filaments in a motility assay were quantitated. AC-Cys\textsuperscript{18}-LC\textsubscript{20} retains an average of 74% of the control actomyosin motility. No motility was observed when phosphorylated wtLC\textsubscript{20}, Cys\textsuperscript{18}-LC\textsubscript{20}, or AC-Cys\textsuperscript{18}-LC\textsubscript{20} were exchanged into unphosphorylated smooth muscle myosin II. Rates are averages and standard deviations from 12-14 filaments. Unexchanged phosphorylated myosin moves actin at a rate of 1.05 ± 0.10 S.D. µm/s (n = 8).

<table>
<thead>
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<th>Exchanged myosin</th>
<th>Velocity µm/s</th>
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<tbody>
<tr>
<td>P04-wtLC\textsubscript{20} (control)</td>
<td>0.84 ± 0.21</td>
</tr>
<tr>
<td>P04-Cys\textsuperscript{18}-LC\textsubscript{20}-myosin</td>
<td>0.88 ± 0.25</td>
</tr>
<tr>
<td>P04-AC-Cys\textsuperscript{18}-LC\textsubscript{20}-myosin</td>
<td>0.62 ± 0.16</td>
</tr>
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wtLC\textsubscript{20}-exchanged myosin (the control) and phosphorylated unlabeled Cys\textsuperscript{18}-LC\textsubscript{20}-exchanged myosin were the same, indicating that changing threonine 18 to a cysteine had no effect on native actomyosin motility. Phosphorylated AC-Cys\textsuperscript{18}-LC\textsubscript{20}-exchanged myosin moved actin at 74% the velocity of the control. It can be inferred from this assay that the actin-activated MgATPase, actin binding, and ability to translocate actin filaments of AC-Cys\textsuperscript{18}-LC\textsubscript{20}-exchanged myosin have not been grossly altered. (Rhodamine-labeled smooth muscle myosin analogs used in live cell experiments retain an average of 80% the actin-activated MgATPase activity of native myosin (15)). AC-Cys\textsuperscript{18}-LC\textsubscript{20} Localizes to the A-band of Chicken Pectoralis Myofibrils and Undergoes a Phosphorylation-dependent Fluorescence Change—AC-Cys\textsuperscript{18}-LC\textsubscript{20} was incorporated into chicken pectoralis myofibrils, as a model in situ “stress fiber” to demonstrate proper light chain localization in the presence of actin and actin-binding proteins. The high affinity of skeletal muscle myosin for smooth muscle light chains (21) was exploited to achieve high efficiency. In addition, skeletal muscle myosin/smooth muscle light chain hybrids are not regulated by wtLC\textsubscript{20} phosphorylation (39) and therefore will not contract upon light chain phosphorylation. Thus, the myofibrils can be used as a simple biological test system in which to quantitate phosphorylation-dependent fluorescence changes with the microscope. Fig. 5 shows that AC-Cys\textsuperscript{18}-LC\textsubscript{20} localizes to the myosin-rich A-bands of the myofibrils. AC-Cys\textsuperscript{18}-LC\textsubscript{20}-exchanged myofibrils were scanned in a fluorometer before and after phosphorylation in order to measure phosphorylation-dependent fluorescence changes. An emission scan of unphosphorylated AC-Cys\textsuperscript{18}-LC\textsubscript{20}-exchanged myofibrils in rigor buffer plus bleaching inhibitors was collected. Phosphorylation reagents (1 µM MLCK, 1 µM CaM, 0.5 mM ATP-S, 1 mM CaCl\textsubscript{2}, and 0.02 unit of hexokinase in rigor buffer) or control reagents (same as above but without MLCK and CaM) were added to the cuvette. After a 1.5-h incubation (with occasional mixing), the sample was rescanned. Phosphorylated myofibrils showed an average of 4.21 ± 0.45 S.D. fluorescence quenching over control, unphosphorylated myofibrils. Urea/glycerol gel electrophoresis of the experimental samples confirmed that the exchanged light chains were ~70% phosphorylated. Values were normalized to 100% phosphorylation to obtain an average fluorescence quenching of 36.4% ± 5.9 S.D. upon phosphorylation of AC-Cys\textsuperscript{18}-LC\textsubscript{20} (Fig. 6).

This experiment was repeated on individual myofibrils using the multimode microscope. The mean fluorescence quenching of 53 control A-bands (n = 19 groups of A-bands) in a preliminary experiment was 31.3% ± 1.4 S.D. and the mean fluorescence quenching of 34 phosphorylated A-bands (n = 14 groups of A-bands) was 43.8%. A t test performed on the two data sets determined these to be two significantly different populations with a p value of <1%. The difference between the means of the unphosphorylated exchanged myosins were able to translocate actin filaments, demonstrating that these myosins are still regulated by light chain phosphorylation. The velocities of phosphorylated myosins were determined as a function of light chain concentration (0-15 mM Ca\textsuperscript{2+}, and 0.02 unit of hexokinase in rigor buffer) the sample was rescanned. Phosphorylated myosin moved actin at a rate of 1.05 ± 0.10 S.D. µm/s (n = 8). Values were normalized to 100% phosphorylation to obtain an average fluorescence quenching of 36.4% ± 5.9 S.D. upon phosphorylation of AC-Cys\textsuperscript{18}-LC\textsubscript{20} (Fig. 6).
phosphorylated myofibrils and the unphosphorylated (control) myofibrils is 12.5%. A parallel sample of AC-Cys\textsuperscript{18}-LC\textsubscript{20}-exchanged myofibrils was phosphorylated with the same phosphorylation reagent concentrations as the above experiment and run on a urea/glycerol gel. AC-Cys\textsuperscript{18}-LC\textsubscript{20} was \textasciitilde70% phosphorylated according to the acrylodan fluorescence under ultraviolet illumination of the gel.

**Fluorescently Labeled Cys\textsuperscript{18}-LC\textsubscript{20} Can Be Incorporated into Stress Fibers of Living Cells—Rhodamine-labeled Cys\textsuperscript{18}-LC\textsubscript{20} light chains were microinjected into A7r5 rat aorta smooth muscle cells (Fig. 7A). The labeled mutant light chains incorporated into the stress fibers of these cells exhibiting the semi-sarcomeric distribution of myosin. Similarly, AC-wtLC\textsubscript{20} was microinjected into Swiss 3T3 fibroblast cells where it incorporated into stress fibers (Fig. 7B). The signal-to-noise ratio of the acrylodan-labeled light chains was very poor in living cells. The 3T3 cells as well as smooth muscle cells were also injected with AC-Cys\textsuperscript{18}-LC\textsubscript{20}, but higher signal-to-noise was obtained from the 3T3 cells. Fig. 7 demonstrates stress fiber incorporation of labeled light chains, but several problems with the AC-Cys\textsuperscript{18}-LC\textsubscript{20} biosensor limit its value in living cells: the low extinction coefficient makes it a very dim probe and the low signal-to-noise requires high irradiance which causes both bleaching of the acrylodan and induced autofluorescence of the cells. We are now screening other environmentally sensitive fluorophores to produce the next generation biosensor for live cell studies.

**DISCUSSION**

**Protein-based Optical Biosensors**—Fluorescent analogs labeled with environmentally insensitive fluorophores are widely used as intracellular tracking agents to quantify the temporal-spatial dynamics of specific macromolecules (12–14, 40). Observing and measuring dynamic changes in chemical activities in living cells has principally depended on the use of various solvent-sensitive fluorescent dyes that act as ion or potential indicators (13, 14). We have merged these two classes of fluorescent reagents to design a new class of fluorescent analogs called protein-based optical biosensors, which are peptides or proteins labeled with environmentally sensitive fluorophores that sense ligand binding events specific to that macromolecule.
MeroCaM was the first protein-based optical biosensor introduced (15, 16). As calcium binds calmodulin, a protein conformational change occurs that exposes a hydrophobic pocket. A merocyanine dye covalently bound to calmodulin near the hydrophobic pocket then enters this domain. The dye exhibits a fluorescence change that is monitored to observe calmodulin activation in living cells. The approach for designing FICRHR (18) was to label the catalytic and regulatory subunits of CAMP-dependent kinase with a different fluorescent dye of an energy transfer pair. Resonance energy transfer occurs in the holoenzyme complex, but when CAMP binds to the regulatory subunit and dissociates the complex, energy transfer is eliminated. Changes in intracellular CAMP concentrations can be measured with this sensor.

Genetically Engineered Optical Biosensor of Phosphorylation—To develop the myosin phosphorylation biosensor, site-directed mutagenesis was employed to move the cysteine of the myosin regulatory light chain from its native "phosphorylation-insensitive" site to a "phosphorylation-sensitive" site, in that the new cysteine can be fluorescently labeled with an environmentally sensitive dye to detect MLCK regulatory light chain phosphorylation. The labeled protein behaves as both a functional regulatory light chain and an indicator of myosin phosphorylation. We have completed the in vitro characterization of the phosphorylation biosensor. This is a valuable "first generation" probe that can provide information in its present form about the timing and spatial distribution of myosin phosphorylation in permeabilized cell studies in muscle and non-muscle systems as well as in reconstituted model systems. Furthermore, the genetic engineering approach taken here can be applied to virtually any cloned protein to design a similar biosensor.

Acrylodan has been used as a polarity-sensitive fluorescent probe by many investigators; the probe demonstrates a red-shifted emission spectrum on exposure to a polar environment and a blue-shifted emission spectrum on exposure to a hydrophobic environment (24, 41-44). We observe a red-shifted emission about the timing and spatial distribution of myosin phosphorylation. The labeled protein behaves as both a functional regulatory light chain and an indicator of myosin phosphorylation. We have completed the in vitro characterization of the phosphorylation biosensor. This is a valuable "first generation" probe that can provide information in its present form about the timing and spatial distribution of myosin phosphorylation in permeabilized cell studies in muscle and non-muscle systems as well as in reconstituted model systems. Furthermore, the genetic engineering approach taken here can be applied to virtually any cloned protein to design a similar biosensor.

A subset of biochemical experiments on myosin (phosphorylation rate, K'ATPase, and actin motility) were chosen to assay the function of both AC-Cys^-LC,M and smooth muscle myosin exchanged with AC-Cys^-LC,M. All assays performed on unla

that microinjected fluorescent gizzard myosin, like endogenous fibroblast myosin, localizes into a semi-sarcomeric punctate pattern along stress fibers (6, 19, 45, 46). Additionally, we have shown that fluorescently labeled gizzard smooth muscle myosin regulatory light chains microinjected into live fibroblast cells localize as does microinjected fluorescent turkey gizzard myosin (15, 19). This technique is an easy in vivo exchange method for the introduction of light chain phosphorylation indicators into living cells.

Future Developments of the Phosphorylation Biosensor—Several weaknesses of the present phosphorylation indicator should be addressed. First, acrylodan has poor spectroscopic properties for live-cell imaging studies. Acrylodan is an ultraviolet-excitatory dye possessing a low extinction coefficient. This requires a very high excitation dose that both bleaches acrylodan quickly and actually causes an increase in cellular autofluorescence upon excitation. (The latter phenomenon is currently under investigation.) We are now designing new sulfur-hydryl-reactive environmentally sensitive long wavelength excitable dyes, such as the merocyanines (16) for improved phosphorylation indicators. Second, AC-Cys^-LC,M exchanged myosin exhibits only a 10-nm wavelength shift upon phosphorylation by myosin light chain kinase. The combination of the low extinction coefficient and UV excitation of acrylodan does not permit us to take advantage of the AC-Cys^-LC,M phosphorylation-induced wavelength shift for ratio imaging, without adding a second, reference fluorophore. Finally, the cysteine mutant was designed in the absence of much regulatory light chain structural information; cysteine 108 was moved to amino acid position 18 with unknown consequences on the protein structure. While we were fortunate that the biochemical properties assayed were nearly that of controls, for future myosin phosphorylation biosensors we plan to apply some of the emerging regulatory light chain structural information to design an improved biosensor.

We view the present protein-based optical biosensor as a prototype phosphorylation indicator, much as "quin2" was a prototype for calcium indicators (47): it works, but important improvements are needed. This myosin phosphorylation biosensor demonstrates the feasibility of designing fluorescent analogs to monitor in vitro protein kinase activity and serves as the foundation for future, improved protein-based phosphorylation indicators of many different proteins.

Acknowledgments—We thank Jim Sellers, who supplied the initial gizzard-purified light chains for preparing labeled light chains and provided helpful discussions, and Susan Lowey, who served as an important catalyst in early discussions about the feasibility of this project. Charlotte Bartosh, Robin DeBiasio, Greg LaRocca, and Judy Monti-beller provided much technical assistance. All members of the Taylor laboratory gave freely of their help and advice, especially Dr. Ken Giuliano. K. T. thanks Thereatha Chatman for excellent technical help.

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Myosin II LC<sub>20</sub> Phosphorylation Biosensor