Table of Contents

Table S1. Characterization of Peptide Thioesters

Peptide Thioester	Sequence	Molecular Formula	Molecular Weight Calculated	$[MH_5]^{5+}$ Calculated	$[MH_5]^{5+}$ Found[a]	HPLC (t_R)[b]
Non-phosphorylated	Ac-DYKDDDDKSSKKAKTKT TKKRPQRATSNVFA-COSBn	$C_{160}H_{261}N_{47}O_{52}S$	3704.89	742.0	742.0	21.3
pSer19	Ac-DYKDDDDKSSKKAKTKT TKKRPQRAT pS NVFA-COSBn	$C_{160}H_{262}N_{47}O_{55}PS$	3784.86	758.0	758.0	21.0
pThr18	Ac-DYKDDDDKSSKKAKTKT TKKRPQRA pT SNVFA-COSBn	$C_{160}H_{262}N_{47}O_{55}PS$	3784.86	758.0	758.0	20.9
pThr18 pSer19	Ac-DYKDDDDKSSKKAKTKT TKKRPQRA pTpS NVFA-COSBn	$C_{160}H_{263}N_{47}O_{58}P_2S$	3864.83	774.0	774.0	20.6
Caged pSer19	Ac-DYKDDDDKSSKKAKTKT TKKRPQRAT cpS NVFA-COSBn	$C_{168}H_{269}N_{48}O_{57}PS$	3933.91	787.8	787.9	22.1
Caged Thiophospho-Ser19	Ac-DYKDDDDKSSKKAKTKT TKKRPQRAT cp(S)S NVFA-COSBn	$C_{168}H_{269}N_{48}O_{56}PS_2$	3949.89	791.0	791.1	22.6

[a] The data were collected by positive ion electrospray ionization mass spectrometry. [b] Retention times were obtained from reverse phase HPLC analytical runs (YMC C_{18}, ODS-A, 5 μm, 4.6 × 250 mm) using the following method: 5% acetonitrile in water with 0.1% TFA for 5 min, followed by a linear gradient of 5-95% acetonitrile in water with 0.1% TFA over 30 min at 1 mL min^{-1}.

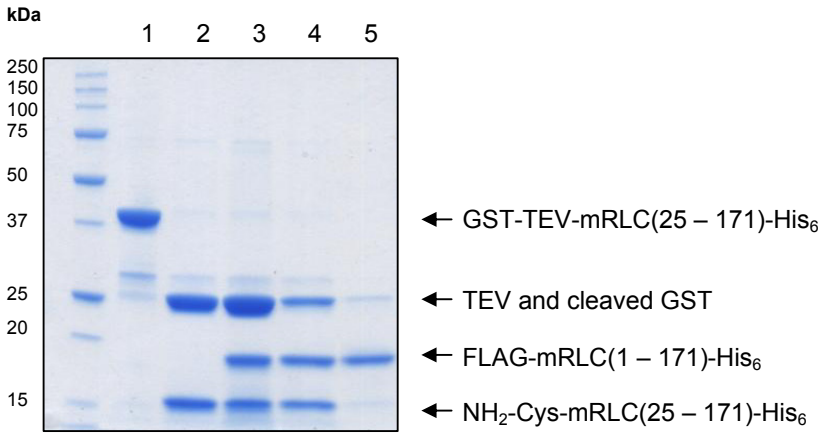

Figure S1. Synthesis and Purification of Semisynthetic mRLC. Coomassie-stained 12% SDS PAGE gel of the mRLC semisynthesis showing GST-TEV-mRLC-His_6 after purification by glutathione resin (*lane 1*), the TEV cleavage of GST-TEV-mRLC-His_6 (*lane 2*), the crude native chemical ligation reaction (*lane 3*), the protein purified by Ni-NTA affinity chromatography (*lane 4*), and the final semisynthetic mRLC after FLAG-affinity purification (*lane 5*).

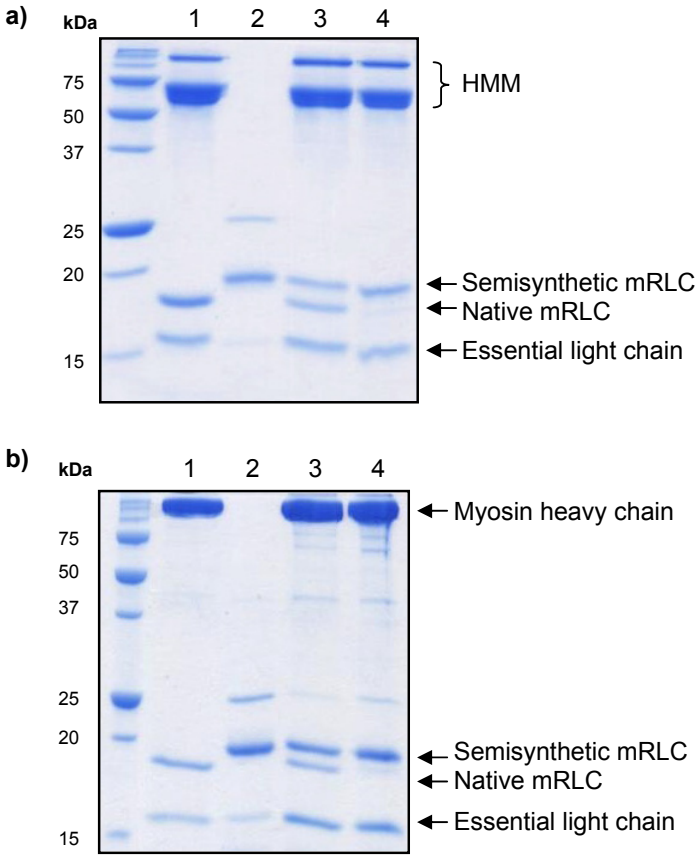

Figure S2. Exchange of semisynthetic mRLC into HMM and Myosin. a) 12% SDS PAGE gel of a representative HMM exchange showing unexchanged HMM (*lane 1*), caged semisynthetic mRLC (*lane 2*), HMM purified after the first exchange (*lane 3*), and HMM purified after the second exchange (*lane 4*). b) 12% SDS PAGE gel of a representative myosin exchange showing native myosin (*lane 1*), caged semisynthetic mRLC (*lane 2*), myosin after the first exchange (*lane 3*), and myosin after the second exchange (*lane 4*). For both HMM and myosin, two consecutive exchanges leads to over 95% incorporation of the semisynthetic mRLC. Due to the addition of the N- and C-terminal tags, the mobility of the semisynthetic mRLC, compared to the native mRLC, is reduced on the SDS PAGE gel.

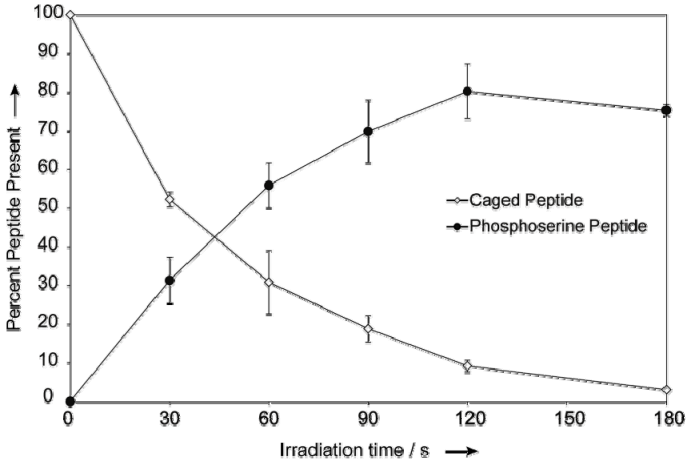

Figure S3. Uncaging time course of caged pSer19 mRLC peptide. A solution of the caged pSer19-mRLC peptide acid (86 μM) in 10 mM HEPES (pH 7.1), 5 mM DTT, and 0.8 μM inosine was irradiated on a transilluminator (365 nm) for the indicated times in a quartz vessel (1 mm pathlength). The peptide species were quantified by analytical RP-HPLC monitored at 228 nm. The areas of the caged and uncaged peptide peaks relative to the area of the inosine peak were determined. The percent of each species relative to the initial amount of the caged peptide is plotted against the duration of irradiation.

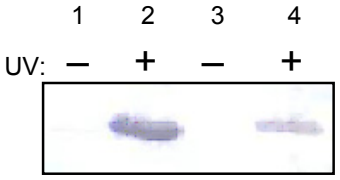

Figure S4. Uncaging of Caged Phosphoserine19 and Caged Thiophosphoserine19 mRLC. Western blot probed with an antibody specific for the pSer19 mRLC showing the caged pSer19 (**5**) and caged thiophosphoserine19 (**6**) mRLCs with no irradiation (*lanes 1 and 3, respectively*) and after 90 s irradiation on a transilluminator (*lanes 2 and 4, respectively*).

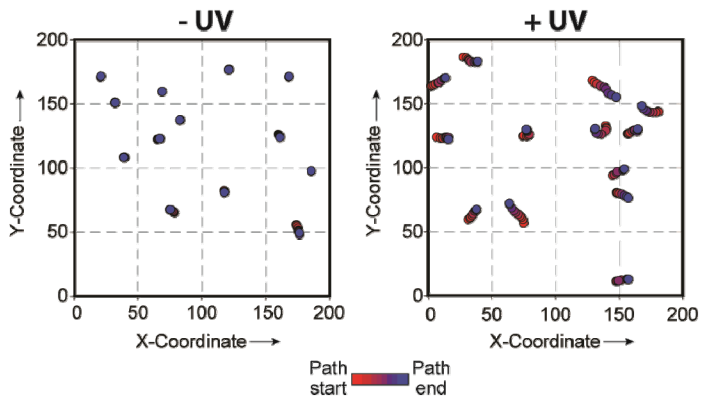

Figure S5. Actin Filament Paths with the Caged Thiophosphoserine19 mRLC. Filament paths in the sliding filament assay with non-irradiated (-UV) and irradiated (+UV) myosin exchanged with caged thiophosphoserine19 mRLC (**6**).

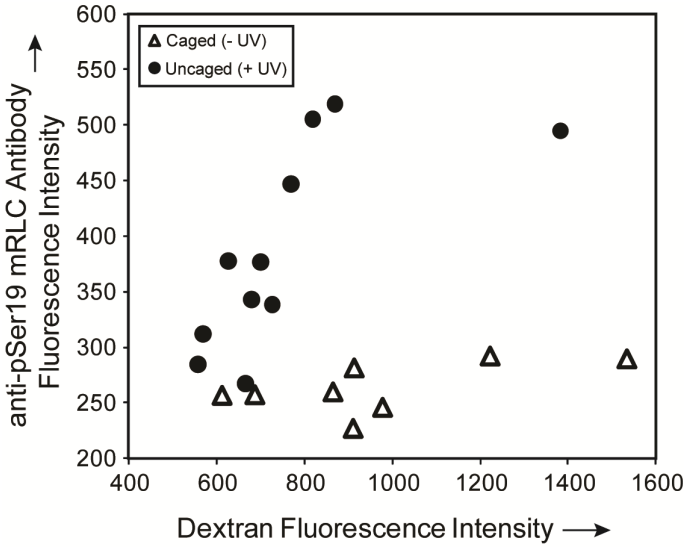

Figure S6. Cellular Uncaging Assessed by pSer19 mRLC Antibody Staining. COS-7 cells were injected with a solution of **6** and Texas Red dextran, exposed to UV irradiation (2 min on a transilluminator) if indicated, fixed, permeabilized, and stained for pSer19 mRLC. The intensity of anti-pSer19 mRLC antibody staining in individual cells is plotted against the dextran fluorescence intensity, which corresponds to the amount of protein that was injected.

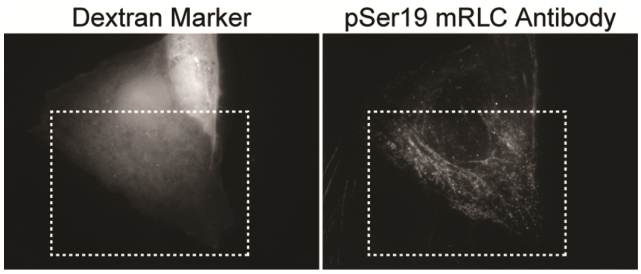

Figure S7. Localized Uncaging within the Cell. COS-7 cells were injected with a solution of **6** and Texas Red dextran and then fixed. A portion of the cell was exposed to UV irradiation under the microscope, and the cells were stained for pSer19 mRLC. The outlined region indicates the portion of the cell that was irradiated.

Materials and Methods

Abbreviations

ATP: adenosine triphosphate; BSA: bovine serum albumin; CIP: calf intestinal phosphatase; DCM: dichloromethane; DIPEA: *N,N*-diisopropylethylamine; DMF: *N*,*N*-dimethylformamide; DTT: dithiothreitol; EDTA: ethylenediaminetetraacetic acid; EGTA: glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid; ESI-MS: electrospray ionization mass spectrometry; Fmoc: 9-fluorenylmethoxycarbonyl; GST: glutathione *S*-transferase; HATU: *O*-(7-azabenzotriazole-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate; HBTU: *O*-benzotriazole-1-yl-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate; HMM: heavy meromyosin; HOAt: 1-hydroxy-7-azabenzotriazole; HOBt: 1-hydroxy-benzotriazole; HPLC: high performance liquid chromatography; IPTG: isopropyl-1-thio-β-D-galactopyranoside; LB: Luria-Bertani; MALDI: matrix-assisted laser desorption ionization; MOPS: 3-(*N*-morpholino)propanesulfonic acid; MWCO: molecular weight cut off; NTA: nitrilotriacetic acid; PBS: phosphate-buffered saline; PyBOP: benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; SDS: sodium dodecyl sulfate; SDS PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBS: tris-buffered saline; TBST: tris-buffered saline with Tween-20; TFA: trifluoroacetic acid; TEV: tobacco etch virus; TIRF: total internal reflection fluorescence; TNBS: 2,4,6-trinitrobenzene sulfonic acid; Tris: tris(hydroxymethyl)aminomethane; TRITC: tetramethyl rhodamine isothiocyanate.

Materials

Unless otherwise noted, all reagents and solvents for peptide synthesis were obtained commercially from Sigma Aldrich and used without further purification. Anhydrous DCM was distilled from calcium hydride. NovaSyn TGT resin, Fmoc-amino acids, PyBOP, HATU, HOAt, HBTU, and HOBt were obtained from Novabiochem. BL-21 Codon Plus RP cells were obtained from Agilent Technologies, Protease Inhibitor Cocktail Set III was from Calbiochem, Glutathione Sepharose 4 Fast Flow was obtained from GE Healthcare, Ni-NTA affinity resin was from Qiagen, and anti-FLAG M2 agarose was obtained from Sigma Aldrich. Amicon Ultra centrifugal filter devices were obtained from Millipore, and Slide-A-Lyzer dialysis cassettes, goat anti-rabbit IgG + IgM (H+L) alkaline phosphatase 2° antibody, and 1-Step NBT/BCIP substrate for alkaline phosphatase were from Thermo Fisher Scientific. Rabbit anti-pSer19 mRLC antibodies for Western blots and cellular studies were obtained from GeneTex and GenScript, respectively. The Alexa Fluor 647 goat anti-rabbit antibody was purchased from Invitrogen. Chicken gizzards and rabbit skeletal muscle acetone powder were purchased from PelFreeze.

Peptide Synthesis

All peptides were synthesized by solid phase peptide synthesis either manually or on an Applied Biosystems 431A peptide synthesizer using Fmoc-protected amino acids. Each peptide synthesis was performed on a 0.04 mmol scale using a 0.2 mmol/g loading Fmoc-Ala-NovaSyn TGT resin, which installed alanine as the C-terminal residue for all peptides. The N-terminus was acetylated by reaction with acetic anhydride and pyridine in DMF (20 equivalents each).

The procedure for the manual synthesis follows. The resin (0.2 g, 0.04 mmol) was swelled in DCM (5 mL) for 5 min and then in DMF (5 mL) for 5 min. The resin was incubated (5 × 5 min) with 5 mL 20% 4-methylpiperidine in DMF and then washed with DMF (5 mL, 5 × 1 min). The next Fmoc-amino acid (0.24 mmol) dissolved in DMF (5 mL) with PyBOP (0.12 g, 0.24 mmol) was added. DIPEA (84 µl, 0.48 mmol) was added, and the reaction was allowed to proceed for at least 45 min.

The success of coupling was evaluated by a TNBS test, and if no beads turned red, the procedure was repeated using the next amino acid. Phosphopeptides were synthesized by employing commercially available Fmoc-Thr(PO(OBzl)-OH)-OH or Fmoc-Ser(PO(OBzl)-OH)-OH. The caged residues *N*-α-Fmoc-phospho(1-nitrophenylethyl-2-cyanoethyl)-L-serine and *N*-α-Fmoc-phosphorothioyl(1-nitrophenylethyl-2-cyanoethyl)-L-serine were synthesized according to Rothman, *et al.*[1] and Aemissegger, *et al.*,[2] respectively. These residues (0.08 mmol) were coupled with HATU (0.08 mmol), HOAt (0.08 mmol), and 2,4,6-collidine (0.10 mmol) to prevent β-elimination of the phosphotriester.

Peptides were prepared by automated solid phase peptide synthesis on an Applied Biosystems 431A synthesizer employing standard Fmoc-protected amino acids (4 equivalents relative to resin loading per coupling), HOBt and HBTU coupling reagents, and 4-methylpiperidine deprotections. Double couplings and acyl capping were performed. On the automated synthesizer, Ser1 and Ser2 were coupled as the corresponding pseudoproline dipeptide Fmoc-Ser(tBu)-Ser($\Psi^{Me,Me}$pro)-OH, and Lys8 and Thr9 were incorporated using Fmoc-Lys(Boc)-Thr($\Psi^{Me,Me}$pro)-OH. These pseudoproline dipeptides improved the yields and purities of the final peptides.

Peptide Thioester Synthesis

The N-terminal acyl-capped peptides (0.04 mmol) were cleaved from the TGT resin without side chain deprotection in 0.5% TFA in DCM for 2 h. The solution was evaporated to about 1 mL, and the peptide was precipitated with hexanes. The solution was rotovapped, and the peptide was dried in vacuo. The peptide was dissolved in freshly distilled DCM (12 mL) under argon. HATU (0.061 g, 0.16 mmol), HOAt (0.022 g, 0.16 mmol), benzyl mercaptan (94 μL, 0.8 mmol), and 2,4,6-collidine (42 μl, 0.32 mmol) were added, and the reaction was stirred at RT under argon for 4 h. Under these conditions, epimerization of the C-terminal alanine was minimized (to ~6% based on model studies). The reaction was then rotovapped to dryness, and the side chain protecting groups were removed in 10 mL of 95% TFA with 2.5% triisopropyl silane and 2.5% H_2O for 2 h. The TFA was evaporated, and the peptide was triturated with cold diethyl ether (40 mL, 3×). Peptides were purified by reverse phase HPLC with a Waters 600 automated control module on a YMC C_{18} semi-preparative column (YMC-Pack ODS-A, 5 μm, 20 × 250 mm) eluting with acetonitrile/water containing 0.1% TFA. For detection, a Waters 2487 dual wavelength absorbance detector was used to record at 228 nm and 280 nm. HPLC conditions were 5% acetonitrile in water with 0.1% TFA for 5 min followed by a linear gradient from 20% to 50% acetonitrile in water with 0.1% TFA over 45 min. Following lyophilization, correct mass was validated by ESI-MS on a Mariner electrospray mass spectrometer (PerSeptive Biosystems) (Table S1). Purity was confirmed by analytical HPLC with a Beckman Ultrasphere C_{18} reverse phase column (YMC ODS-A, 5 μm, 4.6 × 250 mm). Peptides were quantified by absorbance. For caged thioester peptides dissolved in MeOH, ε = 5,700 M^{-1} cm^{-1} at 260 nm. The noncaged peptides were dissolved in H_2O, and absorbance at 280 nm was measured (ε = 1,490 M^{-1} cm^{-1}).

Cloning

To generate the GST-mRLC protein fusion, the C-terminal portion of the mRLC was subcloned into the pGEX-4T-2 vector. The gene fragment encoding mRLC(25 – 171) was amplified by polymerase chain reaction from a vector containing the full mRLC gene (GenBank Accession AK002885). The forward primer for PCR encoded a 5′ *Eco*RI restriction site, followed by the TEV protease cleavage sequence (ENLYFQ) and the Met24Cys mutation, and the reverse primer was used to encode a C-terminal hexahistidine tag and 3′ *Not*I restriction site. The sequences of the primers used for PCR are given below:

Forward primer:
5′-GCCGGAATTCGTGAGAACCTGTATTTCCAGTGCTTTGACCAGTCCCAGATC-3′

Reverse Primer:
5′-GCGAAAGACAAAGATGACCATCACCATCACCATCACTAGGCGGCCGCAAAAGG
GGGC-3′

The PCR amplicons were digested with *Eco*RI and *Not*I and ligated into the pGEX-4T-2 vector, which had been digested with *Eco*RI and *Not*I and treated with CIP. The ligated plasmid was transformed into DH5α cells and grown on LB plates containing carbenicillin (50 μg/mL).

mRLC Expression

The pGEX-mRLC plasmid was transformed into BL21-CodonPlus (DE3)-RP cells, and the bacteria were grown on LB plates containing carbenicillin (50 μg/mL) and chloramphenicol (30 μg/mL). A single colony was selected and grown in LB media (5 mL) supplemented with carbenicillin (50 μg/mL) and chloramphenicol (30 μg/mL). This starter culture was used to inoculate a 1 L culture, which was incubated in a shaker at 225 rpm and 37 °C until an OD of ~0.6 at 600 nm was reached. The culture was cooled to 16 °C, and IPTG was added to 0.2 mM to induce protein expression. The culture was incubated overnight at 16 °C with shaking. The next day, the cells were harvested by centrifugation, and the cell pellets were stored at -80 °C until use.

Isolation and Purification of GST-mRLC

The cell pellet was thawed on ice and brought up in 40 mL of PBS (150 mM NaCl, 10 mM phosphate, pH 7.7) containing 1 mg/mL lysozyme, 1 mM DTT, and 1 μL/mL Protease Inhibitor Cocktail Set III (100 μM AEBSF, 80 nM Aprotinin, 5 μM Bestatin, 1.5 μM E-64, 2 μM Leupeptin, 1 μM Pepstatin A) for each liter of cells harvested. The cells were incubated on ice for 20 min and then sonnicated on ice at 40% amplitude, 1 s on/1 s off for 45 s with a Sonics Vibra Cell sonnicator. Cell debris were pelleted at 90,000 × g for 1 h at 4 °C, and the lysate was passed through a 0.2 μm filter. Glutathione Sepharose 4 Fast Flow (3 mL) was incubated with the cell lysate for 1.5 h at 4 °C. The resin was isolated with a brief centrifugation and washed with 120 mL PBS at 4 °C. The protein was eluted in four 3-mL fractions with buffer containing 10 mM reduced glutathione and 2 μL/mL Protease Inhibitor Cocktail Set III in 50 mM Tris (pH 8.0). The protein was dialyzed in a 3,500 MWCO Slide-A-Lyzer dialysis cassette against PBS (3 × 2 L). Protein concentration was determined through a BioRad assay with BSA as a standard.

TEV Cleavage

TEV cleavage was performed by incubating the GST-mRLC protein (1.6 mg/mL) with TEV protease in buffer containing 50 mM Tris (pH 8.0), 0.5 mM EDTA, and 5 mM β-mercaptoethanol for 3.5 h at 30 °C and then overnight at 4 °C. SDS PAGE confirmed complete proteolytic cleavage.

Native Chemical Ligation

The TEV-cleaved protein (NH_2-Cys-mRLC(25-171)-His_6) was concentrated to 14 mg/mL with an Amicon Ultra centrifugal filter device (MWCO 3,000). Native chemical ligation reactions were performed by combining the thioester peptide (1.2 mM) with the TEV-cleaved protein (0.8 mM) in a buffer containing 150 mM sodium 2-mercaptoethanesulfonate and 50 mM Tris (pH 8.0). The reactions were incubated for 18 h at RT. The mixture was then diluted to 2 mg/mL and dialyzed

against PBS (3 × 2 L) in a Slide-A-Lyzer dialysis cassette (3,500 MWCO) to remove the thiol additives.

Purification of Semisynthetic mRLC

The ligation mixture was purified from excess peptide using Ni-NTA affinity chromatography. The crude ligation was incubated with 2 mL Ni-NTA resin in PBS (25 mM NaH_2PO_4, 25 mM Na_2HPO_4, 300 mM NaCl, pH 7.9) containing 5 mM imidazole. After 1 h at 4 °C, the resin was collected and washed with 120 mL PBS containing 5 mM imidazole. The protein was eluted with 12 mL of PBS containing 300 mM imidazole and dialyzed against TBS (50 mM Tris (pH 7.5), 200 mM NaCl, 3 × 2 L). The unligated protein was subsequently removed from the FLAG epitope-tagged ligation product using anti-FLAG M2 agarose. After incubating the protein with the resin in TBS for 1 h at 4 °C with gentle agitation, the resin was washed with 120 mL TBS. The protein was eluted in 1-mL fractions with 0.1 M glycine (pH 3.5) into 50 μL of a solution of 1 M Tris (pH 7.8) and 0.8 M NaCl. The purification was repeated using the flow through to recover unbound protein. The pooled elutions from each purification were dialyzed into PBS.

MALDI Analysis

Mass analysis of the purified semisynthetic protein was obtained on a Voyager DESTR MALDI by the MIT Biopolymers Laboratory. For semisynthetic nonphosphorylated mRLC **1:** Expected $[MH]^+$: 21479.8; Found $[MH]^+$: 21482.4.

Proteins for ATPase and Sliding Filament Assays

Myosin was isolated from chicken gizzards according to Ikebe and Hartshorne.[3] HMM was generated by myosin proteolysis according to Ikebe and Hartshorne using *Staphylococus aureus* V8 protease, except that a GE Healthcare Superdex 200 HiLoad (16/60) size exclusion chromatography column was used for purification.[4] Myosin light chain kinase was purified from chicken gizzards according to Ikebe, *et al*.,[5] and actin was purified from rabbit skeletal muscle acetone powder following protocols by Pardee and Spudich.[6]

Myosin and HMM Exchange

The semisynthetic mRLC was exchanged into gizzard smooth muscle myosin according to modified procedures by Sherwood, *et al*.[7] and Ikebe, *et al*.[8] Myosin (0.5 mg/mL) in exchange buffer (0.6 M NaCl, 20 mM sodium phosphate (pH 7.5), 10 mM DTT, 5 mM EDTA, 1 mM EGTA, and 5 mM ATP) was incubated for 30 min at 42 °C with about 5 molar equivalents of the semisynthetic mRLC. After cooling on ice, $MgCl_2$ was added to 20 mM. To remove excess light chains, the myosin was precipitated by overnight dialysis into 15 mM Tris (pH 7.5), 10 mM $MgCl_2$, and 1 mM DTT. The pellet was collected by centrifugation and washed with dialysis buffer. The pelleted protein was dissolved in the exchange buffer, and the protein was subjected to a second exchange to increase incorporation of the semisynthetic protein to at least 90%.

The conditions for exchange into HMM (0.5 M NaCl, 20 mM sodium phosphate (pH 7.5), 10 mM DTT, 5 mM EDTA, 1 mM EGTA, and 1 mM ATP) were modified from Ellison, *et al*.[9] The HMM was incubated with a 5-fold excess of the semisynthetic mRLC at 42 °C for 30 min. After cooling on ice, $MgCl_2$ was added to 20 mM, and the excess light chains were purified from HMM through size exclusion chromatography on a GE Healthcare Superdex 200 (10/300 GL**)** column equilibrated in 30 mM Tris (pH 7.5), 300 mM NaCl, 1 mM $MgCl_2$, and 0.5 mM DTT. Fractions containing

HMM were pooled and concentrated in a 50,000 MWCO centrifugal filter unit, and the exchange was repeated.

HMM Phosphorylation by Myosin Light Chain Kinase

HMM was phosphorylated though a modified protocol from Ellison, *et al*.[9] HMM (0.5 mg/mL in 15 mM Tris (pH 7.4), 50 mM NaCl, 2.5 mM $MgCl_2$, and 3.5 mM $CaCl_2$) was incubated with ATP (1.4 mM), myosin light chain kinase (30 μg/mL), and calmodulin (4 μg/mL) for 1 h at RT and then overnight at 4 °C.

Uncaging

Myosin or HMM exchanged with the caged semisynthetic protein in the appropriate assay buffer supplemented with 5 mM DTT was irradiated in a quartz vessel with a 1 mm pathlength on a UVP High Performance Ultraviolet Transilluminator with light centered at 365 nm (7330 μW/cm^2) for 90 s.

Western Blots

Standard SDS PAGE on a 12% polyacrylamide gel was performed, and the proteins were transferred to a nitrocellulose membrane at 100 V for 1 h. The blot was blocked with 5% BSA in TBST (TBS with 0.05% Tween 20) overnight at 4 °C and then incubated with a rabbit anti-pSer19 mRLC antibody (1/1000 dilution) in 3% BSA in TBST for 2 h at RT. The blot was washed in TBST (5 × 5 min) and incubated with a goat anti-rabbit IgG + IgM (H+L) alkaline phosphatase 2° antibody in TBST (1/5000 dilution) for 1 h at RT. The blot was then washed with TBST (3 × 5 min) and TBS (1 × 5 min) and developed with 1-Step NBT/BCIP substrate for alkaline phosphatase.

ATPase Assays

The actin-activated ATPase activity of HMM was determined by measuring the inorganic phosphate released over 30 min.[10] Assay conditions were modified from Ikebe and Hartshorne.[4] HMM (0.1-0.2 mg/mL) in 30 mM Tris (pH 7.5), 2.5 mM $MgCl_2$, 20 mM KCl, 0.1 mM EGTA, and 1 mM DTT was incubated at 25 °C with actin (24 μM) in a final volume of 150 μL. All protein used in the assay was dialyzed into the assay buffer prior to the assay. The assay was initiated by the addition of ATP to 1 mM. For each time point, 30 μL of the myosin solution was added to 30 μL of the stop solution (60 mM EDTA (pH 6.5) with 6.6% SDS). To quantify the amount of inorganic phosphate present in each sample, 120 μL color developing solution (0.5% ammonium molybdate in 1 N H_2SO_4 with 18 mM ferrous sulfate) was added. After incubating the samples at RT for 20 min, the absorbance of the sample at 700 nm was measured. The rate of phosphate release was calculated based on a phosphate standard curve. Enzymatic activity (s^{-1}) was calculated using a molecular weight of 334,000 for HMM.

Sliding Filament Assays

Sliding filament assays were performed according to Sellers.[11] To improve the quality of the actin filament movement, prior to each assay, myosin at 1 mg/mL in 0.5 M KCl, 10 mM MOPS (pH 7.0), 0.1 mM EGTA, 5 mM $MgCl_2$, 2 mM ATP, and 6 μM actin was centrifuged at 480,000 × g for 7 min to remove myosin containing heads that bind actin but that do not hydrolyze ATP. The supernatant was added at a concentration of about 0.2 mg/mL to a flow chamber constructed from a nitrocellulose-coated coverslip and microscope slide. The flow chamber was then blocked with 3

volumes BSA (1 mg/mL) in 0.5 M KCl, 10 mM MOPS (pH 7.0), and 0.1 mM EGTA and then washed with 3 volumes of motility buffer (20 mM MOPS (pH 7.4), 50 mM KCl, 4 mM $MgCl_2$, and 0.1 mM EGTA). Sheared actin (5 μM) with 1 mM ATP in motility buffer was added to block myosin heads that do not hydrolyze ATP, and the flow cell was washed with 3 volumes of motility buffer. TRITC-phalloidin labeled F-actin (20 nM) in motility buffer was added, and the assay was started by the addition of assay buffer (motility buffer containing 1 mM ATP, 20 mM DTT, 0.7% methylcellulose, 2.5 mg/mL glucose, 0.1 mg/mL glucose oxidase, and 20 μg/mL catalase). For native myosin phosphorylated by myosin light chain kinase, the wash containing sheared actin also contained 2 μg/mL myosin light chain kinase, 0.2 mM calmodulin, and 0.2 mM $CaCl_2$. Analysis of the uncaged myosin was performed by irradiating the protein in the presence of 5 mM DTT before adding it to the flow chamber. While movement was observed if the flow chamber itself was irradiated, the quality of the images was compromised due to photobleaching of the TRITC-labeled actin during irradiation. Filament movement was observed with a 100x objective on an Olympus IX50 microscope equipped with a Videoscope ICCD intensified CCD camera and recorded on a Panasonic VHS recorder. Data was quantified using the Cell Tracker software from Motion Analysis and was analyzed according to Homsher, *et al*.[12]

Cellular Experiments

COS-7 cells were cultured in Dulbeco Modified Eagle's Media supplemented with 10% fetal bovine serum at 37 °C in a humidified environment with 5% CO_2. For injection and imaging experiments, cells were transferred to custom-made, glass-bottom pertri dishes made from a No. 1.5 coverslip coated with 2 μg/mL fibronectin overnight at 4 °C. Microinjection needles were pulled from borosilicate glass micropipettes (inner diameter = 0.78 mm, outer diameter = 1.0 mm, with filament) using a Model PC 84 Sachs-Flaming Micropipette puller. Micropipette tip size was estimated to be ~0.5 μm.[13] To prevent the needle from clogging, myosin at ~2.5 mg/mL in sterile PBS supplemented with 295 mM KCl, 0.5 mM DTT, and 1 mg/mL Texas Red-conjugated 10,000 MW lysine-fixable dextran was centrifuged at 75,000 × g for 30 min at 4 °C. The supernatant was then back-loaded into the micropipettes and injected into cells at a pressure of 0.8-1.8 psi using a Narishige IM 300 Microinjector mounted on a Nikon Diaphot microscope. To prevent inadvertent uncaging during injection, a glass UV filter (blocking < 400 nm light) and a red additive dichroic color filter (passing > 600 nm light) were placed in the trans-illumination beam path, and cells were never exposed to arc lamp illumination. For injection, cells were maintained in culture media supplemented with 150 mM KCl to enhance myosin solubility and to prevent the needle from clogging. Post-injection, cells were returned to culture media and allowed to recover for 1-4 h.

Whole-cell uncaging was accomplished by exposing cells to the emission of a Stratagene 2020E transilluminator for 2 min. Cells were allowed to recover for 20 min. The cells were then fixed with 2% formaldehyde and permeabilized with 0.05% saponin. Cells were blocked with a solution of 2% BSA and 0.05% saponin and incubated with a rabbit phospho-specific mRLC antibody diluted 1:400 in blocking buffer. Cells were then blocked and stained with Alexa Fluor 647 goat anti-rabbit antibody diluted 1:400 in blocking buffer. PBS was used as the media during imaging experiments. Cells were imaged on a Nikon TE 300 Microscope equipped with a 100x TIRF lens and a HQ2 Cool Snap Camera. Texas Red-conjugated dextran and Alexa Fluor 647 were imaged through standard filter sets with 500 ms exposures. For experiments with localized uncaging, the cells were fixed following injection. On-scope uncaging was accomplished using the same optical system, but with a pinhole placed in the beam path to allow selective excitation of half of a cell. A 5 s exposure though a high-throughput DAPI filter set (Semrock DAPI-5060B-NQF) was found to be sufficient for uncaging. Antibody staining and imaging were performed as described above.

References

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