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Caged Proteins

Light-Triggered Myosin Activation for Probing Dynamic Cellular Processes**

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Myosin II is an ATPase motor protein essential for many cellular functions, including cell migration[1] and division.[2] During migration of nonmuscle cells, myosin modulates protrusions at the leading edge and promotes retraction at the trailing edge,[3] while during cytokinesis, myosin is required for contraction of the cleavage furrow.[4] For nonmuscle myosin, these varied functions are regulated by phosphorylation of the associated myosin regulatory light chain (mRLC) protein at Ser19, which activates the myosin complex to promote myosin assembly, contractility, and stress fiber formation.[5] These activities are further enhanced upon phosphorylation of the mRLC at both Thr18 and Ser19.[6] The dramatic effects of phosphorylation can also be recapitulated in vitro. Specifically, myosin and the proteolytic derivative heavy meromyosin (HMM),[7] which contains only one-third of the C-terminal myosin tail, exhibit low in vitro activities when associated with the nonphosphorylated mRLC. Phosphorylation of Ser19 amplifies actin-activated ATPase activities 10–1000-fold[8] and leads to myosin-mediated translocation of actin.[9]

While myosin has been studied extensively, questions surrounding the dynamic interactions of the protein in live cells remain. Methods to study myosin and modulate its activity include gene deletions or siRNA-mediated knockdown of gene expression,[3] overexpression of mRLC kinases,[10] and the use of small-molecule inhibitors.[11] While these methods have provided a wealth of information, they do not enable studies of the spatial dynamics of myosin regulation because localized activation cannot be achieved. Additionally, genetic approaches provide imprecise temporal control, thereby preventing real-time studies of protein function. Thus, we sought to develop chemical tools to overcome these drawbacks by enabling the direct and controlled activation of myosin through the semisynthesis of a photoactivated mRLC. The light-mediated activation is achieved by the introduction of a photolabile "caging group" onto the essential phosphate group of pSer19 within the full-length mRLC. The caging group masks the phosphate functionality and renders the protein biologically inactive until irradiation, which releases the native phosphoprotein (Figure 1). The use of light as the trigger for phosphorylation offers a kinase-independent method to activate myosin with precise spatial and temporal resolution, and enables researchers to obtain real-time information about the downstream effects of myosin phosphorylation within a complex network.[12]

For these studies, we chose the 1-(2-nitrophenyl)ethyl (NPE) caging group, which has been employed for cellular applications because it is efficiently released at 365 nm under biologically compatible conditions.[13] Peptides and proteins containing NPE-caged phosphorylated amino acids have been successfully exploited for the study of diverse systems.[14] Additionally, a general method for incorporating NPE-caged thiophosphoamino acids—which, upon irradiation, function like the phosphorylated species but with greater phosphatase resistance—has been reported[15] and can be used for cellular studies of myosin.

Herein we report a chemical approach to investigate myosin function through the preparation of unnatural amino acid mutants of the mRLC. We present an efficient semisynthesis of full-length mRLC through expressed protein ligation for the site-specific incorporation of phosphorylation at Ser19

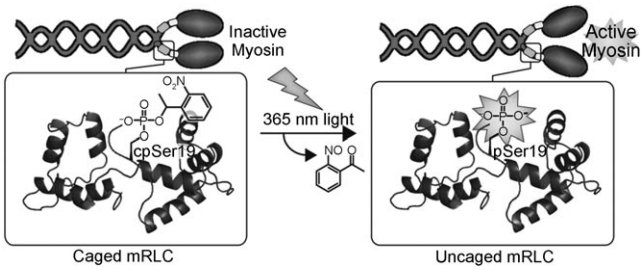

Figure 1. Installation of NPE-caged pSer19 into the mRLC is achieved by expressed protein ligation. The caging group masks the phosphate functionality necessary for myosin activation until irradiation releases the native phosphoprotein to restore activity. The image was modified from Protein Data Bank file 1WDC.

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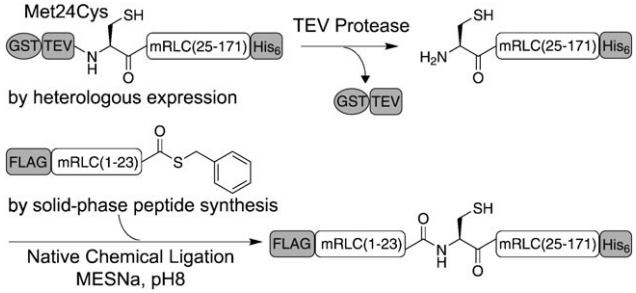
(pSer19) and Thr18 (pThr18), as well as the genesis of caged phosphoserine (cpSer) and caged thiophosphoserine (c(S)pSer) at position 19. Caging of pSer19 eliminates the activity of myosin and HMM, and irradiation releases the native phospho-mRLC to restore activity to nearly native phosphorylated levels. Microinjection of myosin exchanged with the caged protein into live cells and subsequent irradiation liberates the phosphoprotein within the cells. This method is now poised to facilitate investigations of the downstream effects of myosin activation.

Semisynthesis of the mRLC was achieved through native chemical ligation (NCL)[16] between a synthetic peptide thioester corresponding to the N-terminal region of the mRLC (residues 1–23) and a recombinant protein fragment comprising the remaining C-terminal residues (residues 25–171) and a Met24Cys mutation (Scheme 1). To probe the



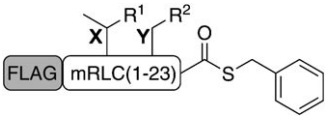
Scheme 1. Semisynthesis of the full-length mRLC. The C-terminal portion of the mRLC is expressed heterologously in *E. coli*. TEV proteolysis releases GST and reveals the N-terminal cysteine residue, which reacts in the NCL with the synthetic peptide thioester to generate the full-length mRLC.

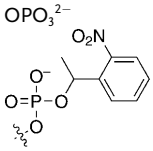
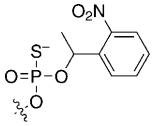
effects of phosphorylation at discrete sites of the mRLC, the protein was synthesized with no phosphorylation (**1**) and with pSer19 (**2**), pThr18 (**3**), pThr18pSer19 (**4**), cpSer19 (**5**), and c(S)pSer19 (**6**).

The peptide thioesters containing the phosphorylated or caged residues were made by solid-phase peptide synthesis (Table 1 and Table S1 in the Supporting Information), and the C-terminal portion of the protein was expressed in *E. coli* as a glutathione S-transferase (GST) fusion to enhance expression and aid purification. Following treatment with the tobacco etch virus (TEV) protease to expose the N-terminal cysteine residue required for ligation, the peptide and protein fragments were combined in the NCL reaction, which efficiently afforded milligram quantities of the full-length mRLC (see Figure S1 in the Supporting Information). N-Terminal FLAG epitope and C-terminal His_6 tags facilitated isolation of the product from unligated protein and excess peptide, respectively. The mass of the protein was confirmed by MALDI analysis.

We characterized the ability of the semisynthetic protein to regulate myosin activity in vitro and to enable myosin photoactivation. Semisynthetic mRLC was exchanged for the native mRLC in chicken gizzard smooth muscle HMM and myosin (see Figure S2 in the Supporting Information), and then tested in ATPase[17] and sliding filament assays.[18] We

Table 1: Peptide thioester derivatives used in the semisynthesis of mRLC.[a]



Entry	Derivative	R^1	R^2
1	nonP	OH	OH
2	pSer19	OH	OPO_3^{2-}
3	pThr18	OPO_3^{2-}	OH
4	pThr18 pSer19	OPO_3^{2-}	OPO_3^{2-}
5	cpSer19	OH	
6	c(S)pSer19	OH	

[a] FLAG-mRLC(1–23): DYKDDDDK-SSKKAKTKTTKKRPQRA***XY***NVFA. Peptides were synthesized by Fmoc-based solid-phase peptide synthesis as C-terminal thioesters. Fmoc = 9-fluorenylmethyloxycarbonyl.

first focused on ATPase assays with HMM due to its greater tractability in solution relative to myosin.[7] Similar to the case of HMM with the native nonphosphorylated mRLC, the actin-activated ATPase activity of HMM exchanged with **1** was negligible (Figure 2a). HMM exchanged with **2** displayed activity similar to that of HMM phosphorylated by myosin light chain kinase (MLCK; (0.80 ± 0.07) and (0.98 ± 0.13) s^{-1}, respectively). These experiments establish that the semisynthetic mRLC faithfully regulates the enzymatic activity of HMM. Additionally, the FLAG epitope and His_6 tags do not influence function.

In addition to Ser19, the mRLC can also be phosphorylated at Thr18.[19] Previous studies of Thr18 phosphorylation alone have relied on a Ser19Ala mutation because Ser19 is

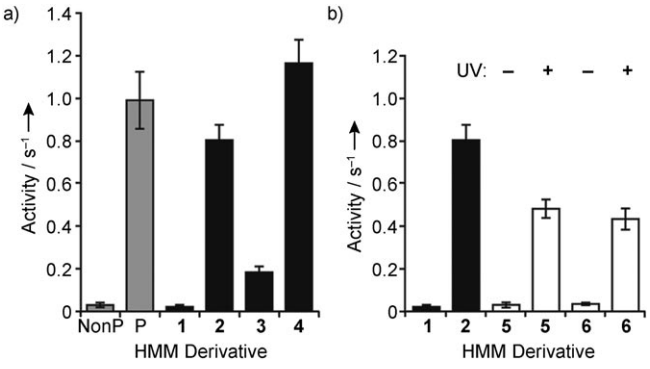


Figure 2. Actin-activated ATPase activities of HMM. The values are the means ± standard deviation (±SD) of at least three trials. NonP: nonphosphorylated; P: phosphorylated by MLCK. a) ATPase activity of HMM with native mRLC (gray bars) and noncaged semisynthetic derivatives (black bars). b) ATPase activity of HMM with semisynthetic noncaged (black bars) and caged derivatives (white bars) before (−UV) and after (+UV) irradiation at 365 nm for 90 s.

normally phosphorylated first.[20] Moreover, mRLC diphosphorylation has been observed in vitro and in cells, but complete in vitro phosphorylation requires high concentrations of MLCK.[19] Our semisynthetic approach provides convenient access to homogeneously phosphorylated proteins, thereby allowing the effects of defined phosphorylation to be examined without the need for additional mutations. ATPase assays of HMM exchanged with **3** showed that phosphorylation of Thr18 moderately increases the activity to $(0.18 \pm 0.03)\ s^{-1}$, whereas phosphorylation at both Thr18 and Ser19 (**4**) generates even greater activity ($(1.16 \pm 0.11)\ s^{-1}$) than pSer19 alone (Figure 2a). These trends are consistent with previous studies on the effects of kinase-mediated Thr18 and Thr18Ser19 phosphorylation.[20]

Next, we investigated the photoactivation of the protein. We used RP-HPLC analysis to examine the kinetics of NPE removal after irradiation of the caged peptide at 365 nm (see Figure S3 in the Supporting Information). Nearly maximal release of the free phosphopeptide (70%) was achieved after irradiation for 90 s, a dosage previously shown to be compatible for cellular studies.[14b] Western blot analysis of the full-length caged proteins (**5** and **6**) with an anti-pSer19 mRLC antibody confirmed that the phospho- and thiophosphoproteins were generated upon irradiation (see Figure S4 in the Supporting Information).

After incorporation of caged mRLCs **5** and **6** into HMM, actin-activated ATPase assays demonstrated that the activity of the caged proteins was low and mimicked that of HMM with nonphosphorylated mRLC **1** (Figure 2b). However, irradiation at 365 nm for 90 s increased the activity about 20-fold to levels near that of HMM exchanged with semisynthetic pSer19 mRLC **2**. Importantly, the caged proteins completely suppress HMM ATPase activity, thus indicating that the NPE group is sufficient to maintain the inhibited state of the protein. The activities following uncaging ((0.48 ± 0.04) and $(0.43 \pm 0.05)\ s^{-1}$ for **5** and **6**, respectively) are consistent with restoration of about 60% of the activity compared to that of HMM with **2** and lie within the range expected on the basis of the HPLC analysis. Thus, irradiation enables direct control over the release of the phosphorylated mRLC and, correspondingly, over the activation of HMM.

To further characterize the system, we performed sliding filament assays, which assess the force-generating ability of myosin. In this assay, we measure the velocities of fluorescently labeled actin filaments propelled by myosin bound to a nitrocellulose-coated glass coverslip. Myosin was used in these assays because it produced more consistent filament movement than HMM. Nonphosphorylated myosin and myosin exchanged with **1** did not move the actin filaments, but both MLCK-phosphorylated myosin and myosin exchanged with **2** led to significant movement, with velocities around $0.9\ \mu m\,s^{-1}$ (Figure 3a). Each phosphorylated semisynthetic derivative generated filament movement at velocities between 0.7 and $1.0\ \mu m\,s^{-1}$. A one-way ANOVA followed by Tukey's post-hoc test indicated that the differences among myosin exchanged with **2**, **3**, and **4** are statistically significant, with all comparisons yielding $p < 0.0001$ (Figure 3a). These results are consistent with a previous study in which myosin with a pThr18Ser19Ala mutant mRLC

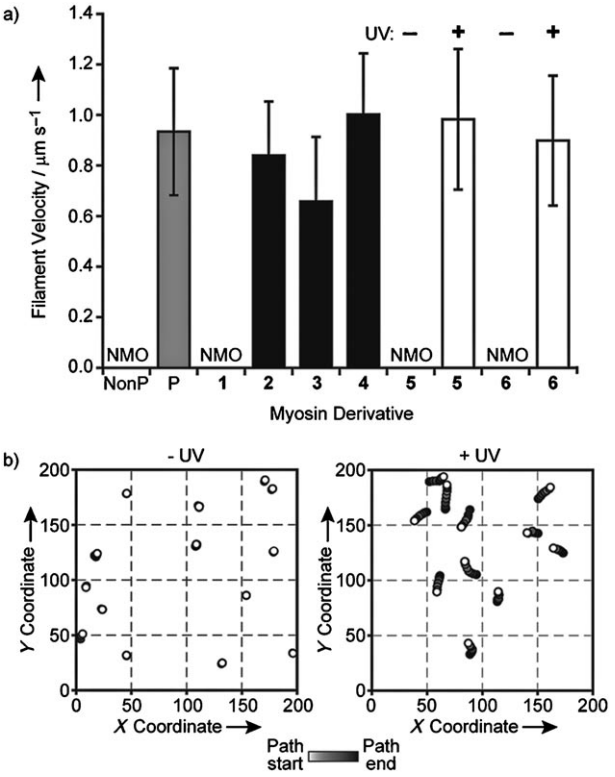


Figure 3. In vitro myosin sliding filament assays. a) The mean velocities ± SD of at least 45 actin filaments during incubation with native myosin (gray bars) and myosin exchanged with noncaged semisynthetic (black bars) and caged semisynthetic (white bars) mRLCs. NMO: no motility observed; NonP: nonphosphorylated; P: phosphorylated by MLCK. b) Actin filament paths from a representative field before (−UV) and after (+UV) 90 s irradiation of myosin exchanged with cpSer19 mRLC **5**.

generated slightly lower filament velocities than the pSer19 and pThr18pSer19 derivatives.[20b] However, our results indicate differences between phosphorylation at Ser19 and diphosphorylation (pThr18pSer19), which have not been previously reported.

Negligible filament movement was observed with both caged proteins **5** and **6** before irradiation (Figure 3 and see Figure S5 in the Supporting Information). In contrast, irradiation of myosin prior to the assay generated significant filament movement, with velocities comparable to those observed with MLCK-phosphorylated myosin or myosin exchanged with **2**. Although about 60% of the HMM ATPase activity was achieved after uncaging, the sliding filament velocities were fully restored following irradiation. Studies have shown that while steady-state ATPase activities increase proportionally with the degree of phosphorylation,[21] sliding filament velocities reach a maximal value even in the presence of nonphosphorylated myosin.[22]

The in vitro studies establish that caging of pSer19 provides effective photochemical control over myosin activity. Finally, to test these chemical tools in live cells, we microinjected the caged mRLC into COS7 cells and investigated uncaging in situ. Initially, the caged thiophosphorylated mRLC **6** was used to minimize potential complicating

effects from cellular phosphatases, thereby eliminating the need for nonspecific phosphatase inhibitors. Since incorporation of the injected mRLC into endogenous myosin complexes was slow, gizzard smooth muscle myosin exchanged with the caged protein was prepared in vitro and microinjected. Following irradiation of the injected cells, the cells were fixed, stained with an anti-pSer19 mRLC antibody, and analyzed by immunofluorescence microscopy. The signal from the anti-pSer19 mRLC antibody was significantly higher following uncaging compared to injected cells that had not been irradiated (Figure 4 and see Figure S6 in the Supporting Information). In addition, irradiation of only a portion of a fixed cell enabled spatial control over the release of activated myosin (see Figure S7 in the Supporting Information). These studies indicate that the thiophosphorylated protein can be readily and reproducibly generated within a cellular system and represent the foundation for future investigations of myosin within living cells.

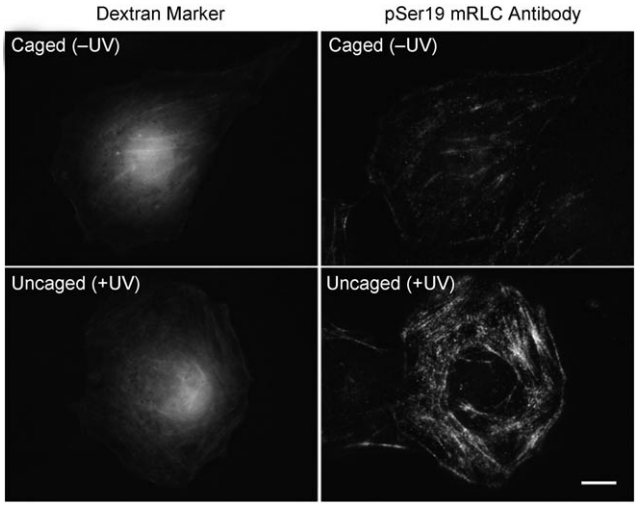


Figure 4. Cells injected with myosin exchanged with **6** and Texas Red labeled dextran marker before (Caged) or after (Uncaged) irradiation. The cells were fixed and stained with an antibody specific for pSer19 mRLC. Scale bar: 10 μm.

In summary, the semisynthetic approach provides convenient access to milligram quantities of various phosphorylated and caged phosphorylated mRLC derivatives to facilitate studies of individual sites of phosphorylation. This general method can be readily adapted to incorporate other unnatural elements into the N-terminal domain of the mRLC. Additionally, the caged protein enables precise photocontrol over the activity of HMM and myosin. Uncaging efficiently furnishes the phospho- and thiophosphoproteins that appropriately regulate the activity of HMM and myosin. The in vitro characterization of the semisynthetic protein and the cellular uncaging experiments provide the basis for subsequent studies of myosin in a cellular context. For example, this system could be used to further address effects of myosin on the formation of stress fibers and focal adhesions. By offering the unique ability to activate myosin with exact spatial and temporal resolution, this approach promises to help unravel the complex role of the protein within the cell.

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