Structural Model of the Regulatory Domain of Smooth Muscle Heavy Meromyosin*

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The goal of this study was to provide structural information about the regulatory domains of double-headed smooth muscle heavy meromyosin, including the N terminus of the regulatory light chain, in both the phosphorylated and unphosphorylated states. We extended our previous photo-cross-linking studies (Wu, X., Clack, B. A., Zhi, G., Stull, J. T., and Cremo, C. R. (1999) J. Biol. Chem. 274, 20328–20335) to determine regions of the regulatory light chain that are cross-linked by a cross-linker attached to Cys108 on the partner regulatory light chain. For this purpose, we have synthesized two new biotinylated sulfhydryl reactive photo-cross-linking reagents, benzophenone, 4-(N-iodoacetamido)-4'- (N-biotinylamido) and benzophenone, 4-(N-maleimido)-4'-(N-biotinylamido). Cross-linked peptides were purified by avidin affinity chromatography and characterized by Edman sequencing and mass spectrometry. Labeled Cys108 from one regulatory light chain cross-linked to 71GMSEAPGPIN81, a loop in the N-terminal half of the regulatory light chain, and to 4RAKAKTKKRPOQR16, a region for which there is no atomic resolution data. Both cross-links were to the partner regulatory light chain and occurred in unphosphorylated but not phosphorylated heavy meromyosin. Using these data, data from our previous study, and atomic coordinates from various myosin isoforms, we have constructed a structural model of the regulatory domain in an unphosphorylated double-headed molecule that predicts the general location of the N terminus. The implications for the structural basis of the phosphorylation-mediated regulatory mechanism are discussed.

The actin-activated ATPase activity and motor properties of smooth muscle and nonmuscle myosins are regulated by phosphorylation of the N terminus of the RLC1 (1–4). The RLC is a subunit of the two head domains (S1) with each S1 containing one motor domain, ELC and RLC. The two S1 domains are attached to a long o-helical coiled-coil domain (tail or rod). The regulatory domain is defined as an RLC and ELC attached to the portion of the heavy chain to which they bind. The unphosphorylated forms of these regulated myosins have low ATPase activity and are unable to move actin filaments, whereas the phosphorylated forms are activated in both respects. Domain requirements for regulation have been elucidated through studies of various proteolytic and expressed subfragments of SMM. HMM, which lacks the C-terminal two-thirds of the tail, is double-headed and regulated (5), but expressed HMMs with truncated tails failed to form double-headed structures and were found to be unregulated (6–8) as was S1 (9–11) and single-headed myosin (12, 13). Therefore, two heads are critical for regulation. Two motor domains were found to be required for regulation of a nonmuscle myosin (14). Most mutant constructs that have altered regulation, appear to have not only an increased ATPase activity in the unphosphorylated state but a decreased ATPase activity in the phosphorylated state. This suggests that the phosphorylated state does not reflect a simple case in which the inhibitory mechanism inherent to the native unphosphorylated state has been removed.

The structural basis of the regulatory mechanisms is unknown. There are no crystal structures of double-headed, and therefore, regulated constructs. There are no atomic resolution data for the N terminus of the RLC (residues 1–24), which includes the phosphorylated serine at position 19 (smooth muscle isoform). The N terminus of the RLC is highly conserved in myosins that are regulated by phosphorylation and has been shown to be critical to the regulatory properties of the molecule (15).

It is likely that the mechanism whereby phosphorylation controls the motor ATPase activity is common and important to other isoforms that are only modulated by phosphorylation or that accomplish regulation through Ca2+** binding. For example, a class of mutations found in the β myosin isoform from cardiomyopathy patients are found clustered near the N terminus of the RLC (16–18) and myosin RLC phosphorylation is a key determinant of the stretch activation response in Dro sophila muscles (19). Ca2+ binding to the ELC turns on molsucan myosins. The Ca2+ ion mediates interactions between

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† The abbreviations used are: RLC, regulatory light chain; ELC, essential light chain; SMM, smooth muscle myosin; HMM, heavy meromyosin; BPIA, 4-iodoacetamido-benzophenone; BBPIA, biotin affinity-tagged BPIA; MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry; u-HMM, unphosphorylated HMM; TLC, thin layer chromatography; DMF, N,N-dimethylformamide; DCC, 1-(3-dimethylamino propyl)-3-ethylcarbodiimide hydrochloride; IAA, iodoacetic acid; THF, tetrahydrofuran; DTT, dithiothreitol; MOPS, 4-morpholino-panesulfonic acid; GndHCl, guanidine HCl; ACN, acetonitrile; tp-HMM, thiophosphorylated HMM; S1, subfragment 1; Asp-N, Glu-C, Arg-C proteolytic cleavage, cleavage on the N-terminal side of Asp, C-terminal side of Glu, and C-terminal side of Arg, respectively.

This paper is available on line at http://www.jbc.org
the RLC and the RLC in addition to the heavy chain (20, 21). Molluscan myosins, like the smooth and nonmuscle isofoms, require two heads for regulation. It is likely that the Ca²⁺-mediated regulatory mechanism and the phosphorylation-mediated regulatory mechanism will have many structural parallels.

The goal of this study was to provide structural information about the regulatory domains of double-headed smooth muscle HMM, including the N terminus of the RLC. In previous work (22) we used a photo-cross-linker, BPIA, to label cysteines in a set of single-cysteine mutants of the RLC, exchange the labeled RLC mutants onto native HMM, and compare the ability of the protein to be photo-cross-linked in two states, unphosphorylated, and thiophosphorylated. For A23C, Q15C and Cys108 we set of single-cysteine mutants of the RLC, exchange the labeled RLC mutants onto native HMM, and compare the ability of the protein to be photo-cross-linked in two states, unphosphorylated, and thiophosphorylated. For A23C, Q15C and Cys108 we.

To a solution of biotin (Sigma, 74.0 mg, 0.70 mmol), diisopropylethylamine (106 mg, 0.82 mmol), and 1-hydroxy-7-

of the method of Gilbert and Rando (25). To a solution of biotin (Sigma, 16.2 mg, 86 µmol) in THF (200 µl), the mixture was stirred for 30 min at 0°C. To this solution was added 1 (10.1 mg, 23 µmol) in DMF (400 µl) and stirred for 30 min. The progress of the reaction was monitored by analytical TLC (MeOH/CCI₄, (20:50)). The mixture was centrifuged in a microcentrifuge, the supernatant was removed, and the product was precipitated by addition of cold H₂O (1.5 ml). The precipitated product was dissolved in DMF (400 µl) and precipitated again by adding cold H₂O (1.5 ml). The supernatant was removed to give 2 (11.8 mg, 86%) as a tan solid. The solid was dissolved in DMF (20–30 ml) and stored at –80°C. DIFP had the ability of the protein to be photo-cross-linked in two states, unphosphorylated, and thiophosphorylated. For A23C, Q15C and Cys108 we.

Flash chromatography (CH2Cl2/MeOH (80:20)) of the material was purified by flash chromatography (CH2Cl2/MeOH (80:20)) to give 1 (381 mg, 76%) as a tan solid: mp 269–271°C.

PARTIAL STRUCTURE OF THE RLC AND THE ELC IN ADDITION TO THE HEAVY CHAIN (20, 21). The ELC did not proceed smoothly without this precaution. Flash chromatography (CH2Cl2/MeOH (80:20)) of the material was purified by flash chromatography (CH2Cl2/MeOH (80:20)) to give 1 (381 mg, 76%) as a tan solid: mp 269–271°C. The ELC did not proceed smoothly without this precaution. Flash chromatography (CH2Cl2/MeOH (80:20)) of the material was purified by flash chromatography (CH2Cl2/MeOH (80:20)) to give 1 (381 mg, 76%) as a tan solid: mp 269–271°C.
exchange (5, 12, 29). Unexchanged RLC was removed by gel filtration (5). [3H]BBPIA-labeled u-HMM was dialyzed to 10 mM MOPS (pH 7.0), 0.1 mM EDTA, 0.05 mM DTT, centrifuged (350,000 × g) at 4 °C for 10 min, and filtered through a 0.45-micron filter prior to irradiation. Ten percent of the sample was retained, and 90% was irradiated as described (29). Samples were lyophilized, dissolved in 6 M GndHCl, 10 mM MOPS (pH 6.5), 1 mM DTT, and 1 mM EDTA, heated to 50 °C for 30 min, and filtered. RLC-RLC dimers (40 kDa) were separated from uncross-linked RLC and heavy chains by gel filtration in the above buffer (two TSK SW4000 columns (Tosohas) in series at 0.2 ml/min at 25 °C). The RLC-RLC dimer was identified on SDS gels and by scintillation counting.

Four Independent Experiments to Purify and Characterize Photo-cross-linked Peptides—In experiment 1, RLC-RLC dimer (11.4 nmol) was digested with 6 μg of endoproteinase Asp-N (Roche Molecular Biochemicals sequencing grade) for 48 h in 0.1 M GndHCl, 50 mM Tris (pH 8.5) at 34 °C, after which another 6 μg of endoproteinase Asp-N was added and allowed to react at 4 °C for another 48 h. NaCl (0.5 M) was added to the sample prior to loading onto a 1-ml neutravidin column (Pierce) equilibrated in 0.1 M GndHCl, 100 mM Tris (pH 8.5), 0.5 mM NaCl, 1 mM DTT, and 1 mM EDTA. The column was washed with the above buffer and 25 mM ammonium bicarbonate, 1 mM DTT. During this wash, 40–45% of the loaded tritium eluted from the column, as was found for all experiments. Further binding could not be achieved with fresh avidin. Biotinylated peptides were eluted with 70% formic acid as buffer recommended by the manufacturer were ineffective. The sample (2.2 nmol) was lyophilized and applied to a C8 reversed-phase column (Brownlee Aquapore, narrowbore) and eluted with a linear gradient of 0.1% trifluoroacetic acid/H2O versus 0.1% trifluoroacetic acid/80% ACN. Radiolabeled peptides (2.4 nmol) eluted over one-third of the gradient. Fractions were lyophilized to near dryness and analyzed by MALDI-MS (Table I).

In experiment 2, an Asp-N digest of RLC-RLC dimers (8.6 nmol) was prepared as in experiment 1. An unirradiated u-HMM (0.86 nmol; not gel filtered) was also prepared. Immobilized neuravidin (Pierce; in a syringe column attached to a pump) was equilibrated with 10 volumes of 50 mM Tris-Cl (pH 8.25 at 25 °C), 0.3 mM GndHCl, 0.5 mM NaCl, 0.5 mM DTT, and 0.5 mM EDTA (loading buffer). The sample (adjusted to 0.5 mM NaCl) was loaded, and the column was washed with 10 volumes of each of the following: 1) loading buffer, 2) loading buffer at 2 M NaCl, 3) loading buffer at 2 M NaCl, 4) 50 mM ammonium bicarbonate, 0.5 mM EDTA, 5) buffer 4 with 0.1% Triton X-100 added, 6) 5 mM ammonium bicarbonate, 7) buffer 6 with 10% ACN, 8) 50 mM sodium acetate pH 4.0, 10% ACN, and 9) H2O. The biotinylated peptides were eluted with 88% formic acid and immediately applied to a Superose Peptide column (Amersham Biosciences; 24 ml, 0.5 ml/min) equilibrated in 0.1% trifluoroacetic acid, 30% ACN (see Fig. 4). Fractions (0.3 ml) were lyophilized prior to sequencing (see Fig. 5) and MALDI-MS (see Fig. 6).

In experiment 3, RLC-RLC dimer (5 nmol) treated with Asp-N as for experiments 1 and 2. EDTA (1 mM) was added to inhibit Asp-N; Glu-C (Roche Molecular Biochemicals sequencing grade; 1/100 w/w) was added and allowed to digest at 25 °C for 3 days. The digest was filtered and loaded onto a 0.5-mL monomeric avidin column (Pierce, Ultralink), and the column was washed as described in experiment 2. The biotinylated peptides were eluted with 70% trifluoroacetic acid. After lyophilization, the sample was dissolved in 200 mM ammonium bicarbonate and clarified by centrifugation. Acylaminoacyl-peptidase (30 μg; Roche Molecular Biochemicals sequencing grade; E.C. 3.4.19.1), EDTA (1 mM), and β-mercaptoethanol (1 mM) were added, and the digest was allowed to proceed for 24 h at 37 °C. The sample was reapplied to a fresh monomeric avidin column and eluted as previously described. After repeated lyophilization the sample was resuspended in 0.1% trifluoroacetic acid/65% ACN to give a precipitate too large to be due to labeled peptides. Therefore the sample was repeatedly centrifuged, and the pellets were washed with 0.1% trifluoroacetic acid/65% ACN. The combined supernatants were lyophilized and treated with Zip-Tips (Millipore). Cysteine thiolate anion was performed with standard peptides (Seq-enzyme Calmix 2 from Applied Biosystems). For experiment 3, masses [M + H]+ were detected in at least 3 of 5 different measurements made on different days on the sample. Values reported are the average ± standard deviations (Table I). Calibration with internal standards resulted in two problems that prevented simultaneous observation of both unknown masses and standards. First, the sample peaks were suppressed to below detection levels, and second, a sample milieu effect caused the measured masses of peptide standards to shift to higher masses (Δ ± 4–5 Da) than was found for standards in the absence of sample. Therefore an external calibration was performed with standard added to the sample. Then, data were acquired on the sample, alone and the external calibration was applied.

RESULTS

Synthesis of Photo-cross-linkers—We have developed two new trifunctional photo-cross-linkers (Fig. 1, compounds 2 and 3) to facilitate purification of photo-cross-linked peptides. They are derivatives of the widely used benzophenone chromophore that forms C–C bonds with polypeptides upon irradiation with UV light (31). We have previously described the synthesis of sulfhydryl-reactive, the benzophenone contains a photoreactive carbonyl. Biotin is an affinity tag.

(v/v) ACN/water with 0.25% trifluoroacetic acid. Data were analyzed using Kaleidograph and MS programs from the Expasy website, FindPept Tool and PeptideMass. Observed masses were identified by comparison to calculated masses generated by summing the masses of photo-cross-linker, parent peptides, and predicted target peptides. We subtracted 18 mass units, since 18 mass units can be lost from benzophenone cross-linked peptides treated under similar conditions (30). Most observed masses matched to this in silico data set.

For experiment 1, three spectra from three different fractions from the C8 reversed-phase column were analyzed on at least two different days. Values reported are the average ± standard deviations (Table I). An external calibration was performed with standard peptides (Seq-enzyme Calmix 2 from Applied Biosystems). For experiment 3, masses [M + H]+ were detected in at least 3 of 5 different measurements made in Table I. Calibration with internal standards resulted in two problems that prevented simultaneous observation of both unknown masses and standards. First, the sample peaks were suppressed to below detection levels, and second, a sample milieu effect caused the measured masses of peptide standards to shift to higher masses (Δ ± 4–5 Da) than was found for standards in the absence of sample. Therefore an external calibration was performed with standard added to the sample. Then, data were acquired on the sample, alone and the external calibration was applied.
Ellison et al. of actin ranged from 0.01 exchanged (data from Ref. 22). Bars (pH 7.0), 0.1 mM EGTA, 2 mM MgCl₂, 1 mM DTT, and 1 mM ATP. The rate of hydrolysis of [\(^{14} \text{C}\)]ATP is in progress.

Assessment of regulation of this and other similarly labeled peptides to justify continuing with the study. A more complete may alter the functional properties of u-HMM to some extent. However, in this case there remains a sufficient level of regulation to justify continuing with the study. A more complete assessment of regulation of this and other similarly labeled HMMs using the more sensitive single-turnover approach (5, 14) is in progress.

Cross-linking Occurs between the Two RLC—Since the regulatory properties of the BBPIA-labeled u-HMM remained largely functional, we expected the photo-cross-linking pattern seen for BBPIA-HMM to be similar to that previously observed for BPIA-HMM (shown to be regulated; (22)). As expected, irradiation caused the formation of RLC-RLC cross-linked dimers but only in the unphosphorylated state. Five different peptides were matched to a parent mass plus an amino acid, but it is not definitive because the yield for serine is often low. Methionine is often targeted by activated benzophenone, presumably at the methylene carbon adjacent to the sulfur (31). In this case the sequencing data suggest strongly that methionine is not targeted. The targeted residue within the 78GPIN81 was in minor amounts relative to the target sequence 66DEYLEGMMSEP GG58. . . .

Experiment 3 was performed to further define the cross-linked regions identified in experiments 1 and 2. An avidin-purified Asp-N/Glu-C/acetyl aminopeptidase digest of RLC-RLC dimers was treated with Zip-Tips without further purification. Two samples were obtained. Edman sequence analysis of the first sample (Fig. 6A) identified the target sequence starting with Gly₇¹ that is contained within the sequences identified from experiments 1 (Table I) and 2 (Fig. 5). Table II shows the MALDI-MS data, which is consistent with the sequencing data. Edman sequence analysis for the second sample (Fig. 6B) shows that the major target peptide was the deacetylated N terminus as the sequences starting with Gly₇¹ was not detected even though they were detected in the MALDI-MS spectra (Table II). Data from experiment 3 are consistent with data from Table I and Fig. 5 and suggest that GMS⁷¹, G/GPIN⁸¹, and the first 25 residues from the N terminus are targeted. From Fig. 5 it appears that Ser⁷⁴ is a cross-linked amino acid, but it is not definitive because the yield for serine is often low. Methionine is often targeted by activated benzophenone, presumably at the methylene carbon adjacent to the sulfur (31). In this case the sequencing data suggest strongly that methionine is not targeted. The targeted residue within the 78GPIN81 was not identified, but the sequencing data suggest that it is Asn⁸³.

In experiment 4, a tryptic digest was performed to identify targeted residues from the N terminus by MALDI-MS (Fig. 7). Other previously identified targeted regions were not observed in this experiment because the peptides were too large. Five different peptides were matched to a parent mass plus an arginine residue. Arg⁴ is the only single arginine predicted from a tryptic digest and is therefore a targeted residue. A lysine residue was also targeted and is most likely Lys¹², but Lys¹⁵⁰ was also possible. The dipeptide AK could be residues 5–6 and/or 7–8. By combining the information from Figs. 6B and 7, we can be reasonably sure that Ser¹, Ser², Ala⁵, Ala⁶, Thr⁷, and Gln¹⁵ are not targeted. Lys⁸, Lys⁹, and Thr²⁵ are potentially targeted, and Arg⁴, Lys⁸, Lys¹¹, Lys¹², Arg¹³, Pro¹⁴, and Arg¹⁶ are probably targeted. Therefore, the region RAKAKTKKRPQR¹⁶ can approach within cross-linking distance of benzophenone.

**Discussion**

Summary of Target Peptides and Residues—By analysis of MALDI-MS and Edman sequencing data from four independent photo-cross-linking experiments, we have shown that Cys¹²⁵ of an RLC must be near two general regions of the partner RLC in u-HMM; the region including 71GMS⁷¹ and 78GPIN⁸¹ with Ser⁷⁴ and Asn⁸³ as likely targeted residues, and the region RAKAKTKKRPQR¹⁶ with Arg⁴, Arg⁶, Lys¹¹, gel-filtered (Fig. 4). For the unirradiated sample, most of the radioactivity eluted in fractions 35–40 that contained parent peptides. For the irradiated sample, the major portion of the radioactivity eluted earlier in the profile, as would be expected for the larger parent-target cross-linked peptides. Edman sequencing was performed for 15 cycles on fractions 6–8 from Fig. 4 (irradiated sample), and two major peptide sequences were observed (Fig. 5); the parent peptide that was also detected in the MALDI-MS spectra from experiment 1 ([M + H]⁺ = 1634.92), and the target sequence 66DEYLEGMMSEP GG58. . . . were found in approximately equal quantities, strongly suggesting that the peptides were cross-linked together. The acetylated N terminus did not sequence. The sequence 49DKE⁵¹ was not detected in Fig. 5, but it was observed in the MALDI-MS (peptides 16–21 from Table I). Together these data suggest that, while present, the sequence 49DKE⁵¹ was in minor amounts relative to the target sequence 66DEYLEGMMSEP GG58. . . . .
These regions are not cross-linked in tp-HMM. No match or an ambiguous match was found for the following observed masses: 3775.80 (to account for dehydration). Adding 478.573 (for BBPIA) to the calculated average mass of the unlabeled parent.

The labeled parent peptides used for matching were DVIRNAFAC(bz)F and EDVIRNAFAC(bz)FD. No match was found to calculated masses for unlabeled RLC or labeled RLC (not cross-linked). No match or an ambiguous match was found for the following observed masses: 3775.80 (to account for dehydration). Adding 478.573 (for BBPIA) to the calculated average mass of the unlabeled parent.

The calculated average mass of the labeled parent [M + H]+ plus the attributed target minus 1 (to account for two [M + H]+ added) minus 18 (to account for dehydration).

The labeled parent peptides used for matching were DVIRNAFAC(bz)F = 1634.92, DVIRNAFAC(bz)FD = 1750.01, EDVIRNAFAC(bz)F = 1764.92, DVIRNAFAC(bz)FD = 1857.12, DPEDVIRNAFAC(bz)F = 1976.23, DPEDVIRNAFAC(bz)FD = 2091.12. It was assumed that Asp-N protease cleaved on the N-terminal side of both D and/or E, and up to two missed cleavage sites could be present. The (bz) designation indicates cysteine labeled with BBPIA. Masses of labeled parent peptides were calculated by adding 478.573 (for BBPIA) to the calculated average mass of the unlabeled parent.

Sequences found to match to the calculated masses, assuming cleavage on the N-terminal side of D and E and up to two missed cleavage sites.

AcS is acetylated serine.

Hydrated form (18 mass units not subtracted).

Lys12, Arg13, Pro14, and Arg16 as likely targeted residues. These regions are not cross-linked in tp-HMM.

Products of Protein Photo-cross-linking with Benzophenone Derivatives—Our study has revealed new mechanistic information about benzophenone photochemistry. It is known that the product of benzophenone cross-linked to the α-carbon of glycine can dehydrate (31) and that peptides containing a targeted methionine exist in a form that is 18 mass units lighter than expected (30). Most of our cross-linked peptides were 18 mass units lighter than predicted (31). For example, peptide 1–25 was found in the dehydrated form (Table I). This peptide does not contain glycine and we have no evidence for cross-linking of methionine. We identified Lys and Arg as targeted amino acids in this region. This suggests that these amino acids are also prone to dehydration as may be Ser and Asn (Tables I and II). We do not know if this dehydration occurs during the photolysis.
or in the mass spectrometer. Most likely, a proton on the methylene carbon adjacent to the heteroatom of the side chain leaves as water along with the OH of the biphenyl alcohol of benzophenone after C–C bond formation, thus forming a double bond. This new information should alert investigators to analyze MS data considering both hydrated and dehydrated forms of cross-linked peptides, as we have here.

Effect of BBPIA upon Regulatory Properties of HMM—Labeling of Cys108 with the bulky BBPIA, unlike the smaller BPIA (22), minimally altered regulation (Fig. 2). In contrast, labeling of Q15C and A23C with BBPIA significantly disrupted regulation (data not shown), whereas regulation was intact with BPIA (22). This suggests that these residues are positioned in critical locations important for regulation.

Motor Domains Are Not Required for the Phosphorylation-dependent Structural Changes—We have previously shown (22) that the HMM cross-linking pattern was not altered by ADP or ATP. And we showed that RLC-RLC cross-linking occurred in an unphosphorylated construct lacking motor domains and that phosphorylation abolished the cross-linking. Therefore, it appears that our experiments are sensing a phosphorylation-induced conformational change in the RLC that does not require motor domains. Similarly, Rosenfeld et al. (33) showed that RLC rotational motion in a construct lacking the motor domains is increased by RLC phosphorylation. None of the interactions in Table III were observed in tp-HMM in any nucleotide state.

Structural Model of u-HMM Regulatory Domain Structure—Table III summarizes data we considered to develop a computational model of the u-HMM regulatory domain. The general features of the model are shown in Fig. 8, the details of which will be published elsewhere. It describes the relative orientation of the regulatory domains during the cross-linking event. To build this symmetrical model both benzophenone moieties (one from each RLC Cys108) were locked within 1.4 Å of Gly78 of the other head, and the structure was adjusted to avoid Van der Waal's overlap and to agree with Table III. No
Table II

Analysis of MALDI-MS data from experiment #3

| Observed$^a$ | Calculated parent-target | Calculated parent | Attributed target sequence | Target residues |
|--------------|--------------------------|-------------------|----------------------------|----------------|
|              | [$M + H]^{+}$             | [$M + H]^{+}$     | [$M + H]^{+}$               |                 |
| 4945.75 ± 1.95 | 4945.66                  | 1750.01           | AcSSKRAKAKTTKKRPQRATSNVFAMFDQGS(Q) | 1–28 |
| 4675.78 ± 1.95 | 4673.48                  | 1519.83           | SSKRAKAKTTKKRPQRATSNVFAMFDQGS(Q) | 1–28 |
| 2834.17 ± 0.15 | 2835.31                  | 1750.01           | (EGMMSEAPGPINF)             | 71–81 |
| 2719.19 ± 0.36 | 2720.52                  | 1648.92           | (EGMMSEAPGPINF)             | 71–81 |
| 2719.19 ± 0.36 | 2721.19                  | 1750.01           | (EGMMSEAPGA(N)             | 71–80 |
| 2548.87 ± 0.06 | 2548.06                  | 1519.83           | (GMSEAPGPINF)              | 72–81 |
| 2433.79 ± 0.35 | 2432.79                  | 1879.12           | (EGMMSE/A$^b$              | 71–75 |
| 2318.85 ± 0.34 | 2317.66                  | 1764.03           | (EGMMSE/A$^b$              | 71–75 |
| 2132.55 ± 0.24 | 2131.46                  | 1750.01           | EAPGPINF$^c$              | 76–81 |

$^a$ Observed masses from two Zip-tip treated samples from experiment #3. All peptides were found in both samples (Fig. 6A and 6B), except the first two which were found only in the sample from Fig. 6B. AcS is acetylated serine. No match was found to unlabeled RLC or labeled RLC (not cross-linked) peptides. No match was found to the following masses: 3221.54 ± 0.30, 3134.57 ± 0.75, 2903.81 ± 0.67, 1675.73 ± 0.40, 1576.73 ± 0.48, 1548.99 ± 1.05. All calculations are as in Table I. Parent sequences were the same as in Table I, except that VIRNAFAC/30E = 1519.83 was also included because it was observed in the spectra.

$^b$ Hydridated form of the peptide.

FIG. 7. Peptides in N-terminal region identified by MALDI-MS (experiment 4). The first 16 residues of the RLC sequence are shown. Each line spanning the residues represents an independent measurement of a mass matching that region. Darker lines represent more than one observation. Dotted lines mean that the match could be in more than one place; either of two AK positions, respectively. The position of the lines above the sequence is not important.

SSKRAKAKTT KKRQPQR

Other symmetrical models were consistent with the data in Table III. The two RLC are side by side in an antiparallel manner. Fig. 8 is not meant to indicate specific RLC interactions, but merely suggests their relative orientation and separation during the cross-linking event. The two Phe$^{29}$ (the most N-terminal residues for which we have atomic resolution data) are close together at the interface between the two RLCs in the center of Fig. 8A and in the lower portion of the RLCs in Fig. 8C.

To test our model, we performed an additional cross-linking experiment. Irradiation of BPIA-labeled T83C HMM formed RLC-RLC dimers, only in the unphosphorylated state, and nucleotide did not appear to affect the result (data not shown). This result is consistent (Fig. 8) as Thr$^{53}$ is positioned at the top of the groove between the two RLC within cross-linking distance to the partner RLC.

Model Predicts Location of RLC N Terminus—Our model allows us to predict the position of the first 24 RLC residues, a region for which there is no atomic resolution data. This area is of particular interest because it contains the critical regulatory phosphorylation site at Ser$^{19}$. First, we generated an independent model of these first 24 residues by using secondary structure and disorder prediction tools and analysis of kinase structures with bound substrate peptides. Our model predicts that Ser$^1$ to Thr$^{10}$ or Lys$^{14}$ forms a helix followed by a disordered region that cannot be assigned secondary structure. This latter portion could maximally extend −32 Å from Lys$^{11}$ to Met$^{24}$. Fig. 8B shows the independently modeled N-terminal 24 residues (gray) from the lower RLC (blue) placed onto the regulatory domain model in a manner consistent with our data. We have shown that residues within 4RAKAKTTKKRPQR$^{16}$ can approach within 8–9 Å of the Cys$^{106}$ sulfur of the partner RLC (Fig. 6B, Tables I and II, and Fig. 7). All the probable and potentially targeted residues found within residues 1–11 (Fig. 7), a region that we have predicted to be a helix, are located on one face of such a helix. This suggests that residues 1–11 may be folded into a helix. However because so many residues within this region were targeted, the helix and the benzophenone are not highly restricted in space relative to one another. Much of the N terminus including Ser$^{19}$ (red) lies in a groove between the two RLC. This placement may explain the fact that we observed altered regulation in A23C and Q15C mutants labeled with a bulky group. Previous experiments with the less bulky BPIA-labeled Q15C and A23C on u-HMM (22) showed that RLC-RLC dimers were formed upon irradiation, but the site of labeling was not identified. This model places these two residues, flanking the Ser$^{19}$ (pink), within cross-linking distance of the partner RLC. As seen in Fig. 8B, the N terminus interacts with the C-terminal domain of the partner RLC. Several studies have shown that elements of the C-terminal domain are crucial to proper regulatory properties (34–36). It is tantalizing to suggest that the N terminus is positioned to control the conformation of the heavy chain helix to which the two RLC bind. It has been previously suggested that Ca$^{2+}$ binding in up-regulated scallop myosin may play a role in stabilizing the regulatory domain through tightened interactions between the RLC, ELC, and heavy chain (21). The N terminus may also be strategically placed to control the interface between the two RLC and the attitude and flexibility of the linker connecting the two domains of the RLC. This latter region is known to be important to the regulatory mechanism (37) and has been noted to be different between the skeletal and scallop structures (21).

Model Predicts Phosphorylation-induced Motion of RLC N Terminus—Phosphorylation must result in movement of Cys$^{106}$ out of cross-linking distance to the 71GMSEAPGPIN$^{81}$ and 4RAKAKTTKKRPQR$^{16}$ targets of the partner RLC as photo-cross-linked RLC-RLC dimers were not observed in t-HMM. It may be that movements of the two targets are consequences of one another. Our model shows that cross-linking could occur from Gln$^{15}$ and Ala$^{23}$ to the partner RLC, in agreement with Table III. However, upon phosphorylation, neither Gln$^{15}$ or Ala$^{23}$ can cross-link to the partner RLC suggesting a phosphorylation-induced movement of a significant portion of the N terminus. This could occur if the phospho-serine folded back the N terminus upon itself through coordination with its or other basic residues. In our model, the phosphorylated serine is in a strategic position to dramatically alter the interactions of the N terminus with such a coordination. It is interesting that phosphorylation sites are often in regions of known disorder (38), and we predict that the region around Ser$^{19}$ is disordered. Disorder is also common in protein-protein interactions that are part of regulatory switches (38). The role of phosphorylation may be to transition this region of the N terminus to...
Does Phosphorylation Dissociate Regulatory Domains?—Our model is consistent with previous work showing that isolated u-SMM regulatory domains dimerize (33) and further suggests that interactions between the two RLC provide the stabilizing forces. However, phosphorylation has little effect upon the stability of the dimers (33). Our results using zero-length cross-linking were consistent with these findings.2 Rosenfeld et al. (33) showed that the rotational motion of a fluorophore on Cy518 in a construct lacking motor domains is increased by RLC phosphorylation. However, the rotational motion was slower than expected for mobile regulatory domains moving independently of the rods. In light of this, the transition we observe from cross-linking in the u-HMM to no cross-linking in the tp-HMM may be the result of local motions that do not destabilize RLC interactions. Our data, which shows that the N terminus moves out of cross-linking distance to Cy518, may be sufficient to explain the changes in rotational motion observed by Rosenfeld et al. (33).

Implications of the Model for S2 Structure—The distance between the C termini of the heavy chains at the head-rod junction is 47 Å in our model, suggesting that the rod cannot adopt a coiled-coil up to the head-rod junction. We were unable to find any satisfactory models with such a coiled rod conformation. Our model is consistent with the idea that optimal mechanical performance of SMM requires the rod to uncoil near the heads (39).

Comparison to Other Models—The data from Table III do not fit a model developed from a three-dimensional reconstruction of frozen hydrated expressed smooth u-HMM on a lipid bilayer (40). Neither can RLC Cy518 reach RLC residues 71–81 from either head, nor are inter-head Cy518 to N termini interactions likely without large rearrangements. Since the rod interacts with the lipid bilayer and the heads are arranged on top of the rod, it may be such an interaction stabilizes a structure not found in solution.

We previously reported (22) that our cross-linking data did not fit a computed model (24) of scallop myosin regulatory domains (atomic resolution data from Xie et al. (20) in the presence of Ca2+) attached to a model of the C-terminal portion of the u-helical coiled-coil S2 (tail) domain. The data presented here reinforce that conclusion. To generate our model from the scallop model, the coiled-coil must be unwound to allow the opposite faces of the RLC to interact. We are unaware of any structural data in the scallop system that supports this computed model.

SMM 10 S Studies Support the Model—The 10 S or folded conformation of u-SMM, like u-HMM, is kinetically inactive. Several studies have shown that the N terminus of the RLC is required to form a 10 S SMM structure (41, 42) with the tail folded onto the heads. However, myosin containing an N-smooth/C-skeletal RLC chimera, which contains a complete N terminus, also fails to fold to 10 S (34). This suggests that an interaction of the N terminus with the C-terminal domain may be critical to generate a binding site on the heads for the tail in 10 S. Since single-headed myosin fails to form the 10 S (29, 41)
it appears that elements from both heads are required to generate this binding site. We have previously shown (29) that BPIA-labeled Cys108 photo-cross-links to the tail in the 10 S conformation. All these data point to a structure where the RLC N terminus interacts with the RLC C-terminal domain on the partner head and that this interaction is required for tail interaction near Cys108. These data taken together are highly consistent with our model, which places Cys108 in the RLC C-terminal domain close to the N terminus of the partner RLC. We propose that an interaction similar to that in Fig. 8 is consistent with our model, which places Cys 108 in the RLC C-terminal domain close to the N terminus of the partner RLC. We thank Dr. Rob Ronald, Dr. Yin Luo, and Dr. Thomas Bell for helpful discussions and technical assistance. We thank Derek Pouchnik for zero-length cross-linking results. We are grateful for RLC antibody from Dr. James Stull and Dr. Kristine Kamm.

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