Phosphorylation-dependent Structural Changes in the Regulatory Light Chain Domain of Smooth Muscle Heavy Meromyosin*

(Received for publication, February 12, 1999, and in revised form, April 7, 1999)

Xiangdong Wu‡, Beatrice A. Clack§, Gang Zhi¶, James T. Stull¶, and Christine R. Cremo‡

From the ‡Department of Biochemistry and Biophysics, Washington State University, Pullman, Washington 99164-4660 and the ¶Department of Physiology, University of Texas Southwestern Medical Center, Dallas, Texas 75235-9040

Smooth muscle heavy meromyosin, a double-headed proteolytic fragment of myosin lacking the COOH-terminal two-thirds of the tail, has been shown previously to be regulated by phosphorylation. To examine phosphorylation-dependent structural changes near the head-tail junction, we prepared five well regulated heavy meromyosins containing single-cysteine mutants of the human smooth muscle regulatory light chain labeled with the photocross-linking reagent, benzophenone-4-iodoacetamide. For those mutants that generated cross-links, only one type of cross-linked species was observed, a regulatory light chain dimer. Irradiated mutants fell into two classes. First, for Q15C, A23C, and wild type (Cys-108), a regulatory light chain dimer was formed for dephosphorylated but not thio-phosphorylated heavy meromyosin. These data provide direct chemical evidence that in the dephosphorylated state, Gln-15, Ala-23, and Cys-108 on one head are positioned near (within 8.9 Å) the regulatory light chain of the partner head and that thiphosphorylation abolishes proximity. This behavior was also observed for the Q15C mutant on a truncated heavy meromyosin lacking both catalytic domains. For the actin-heavy meromyosin complex, cross-links were formed in both de- and thio-phosphorylated states. S59C and T134C mutants were in a second mutant class, where regulatory light chain dimers were not detected in dephosphorylated or thio-phosphorylated heavy meromyosin, suggesting positions outside the region of interaction of the regulatory light chains.

The actin-activated ATPase activity and motor properties of smooth muscle and nonmuscle myosins are regulated by phosphorylation of the regulatory light chain (1–3). The dephosphorylated forms of these regulated myosins have low ATPase activity and are unable to move actin filaments, whereas phosphorylated forms are activated in both respects. Domain requirements for regulation have been elucidated through studies of various proteolytic and expressed subfragments of SMM.1 SMM contains two head domains (S1) attached to a long α-helical coiled-coil domain (tail). Single-headed myosin (4) and S1 (5, 6) are active in both dephosphorylated and phosphorylated states. HMM that lacks the COOH-terminal two-thirds of the tail is double-headed and well regulated (5, 7), but expressed HMMs with shorter tails failed to form double-headed structures and were found to be unregulated (8, 9) as in S1 and single-headed myosin. Therefore, two heads are critical for down-regulation, suggesting that head-head interaction is an important feature of the dephosphorylated state. However, a double-headed structure may not be sufficient for full regulation, and interactions between heads and rods may also be required (10).

The general location of the phosphorylated subunit (RLC) at the junction between the heads and tail (11) suggests that RLC-RLC interactions may be a logical consequence of head-head interactions. Indeed, the RLC has been shown to be critical to the regulatory mechanism. In particular, disruption of the COOH-terminal domain (12, 13), the central helix (14), and portions of the NH2-terminal charged region (15) of the RLC alters regulation. Smooth muscle myosin with exchanged skeleton RLC was not activated by phosphorylation (16). In contrast to the RLC, the ELC appears to be less important to regulation because it can be replaced by the skeletal isoform or removed entirely with little effect on regulation (17). The requirements for full regulation in smooth muscle myosin are complex, because of a subtle interplay between the light chain domain, the actin binding loop (18), the catalytic domain (19), and the heavy chain sequence within the light chain domain (20).

The exact location of the phosphorylated serine (S19 in chicken gizzard) of the RLC is not known. The analogous residue was found to be within a disordered region of the NH2 terminus in the crystal structures of both skeletal S1 (21) and the light chain domain of scallop myosin (22). It is possible that this region adopts an ordered structure in a double-headed molecule. Clearly, more information about the structure of the head-tail junction including the RLC is needed to understand the phosphorylation-dependent regulatory mechanism.

Our approach has been to use direct chemical methods to further define this region by looking for conformational differences between the dephosphorylated and phosphorylated forms of smooth muscle HMM. Specifically, we have placed photocross-linking probes selectively throughout the RLC by use of a set of single cysteine mutants. We have labeled two residues within the putative flexible NH2-terminal region (Q15C and A23C), two within the COOH-terminal domain (Cys-108 and T134C), and one within the NH2-terminal domain (S59C). After exchange of the labeled RLC into HMM, we found that cross-linked RLC-RLC dimers were formed after irradiation of Q15C, A23C, and WT

* This work was supported in part by National Institutes of Health Grants HL26043 (to J. T. S.) and AR40917 (to C. R. C.) and by funds from the American Heart Association (to C. R. C.). 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 1754 solely to indicate this fact.

‡ Postdoctoral Fellow of the American Heart Association (Washington State Affiliate).

§ To whom correspondence should be addressed: Dept. of Biochemistry and Biophysics, P. O. Box 644660, Washington State University, Pullman, WA 99164. Tel.: 509-335-2428; Fax: 509-335-9888; E-mail: cremo@wsu.edu.

¶ The abbreviations used are: SMM, smooth muscle myosin; HMM, heavy meromyosin; RLC, regulatory light chain; ELC, essential light chain; BP-RLC, RLC labeled on cysteine with benzophenone-4-iodoacetamide; HisRLC, His6-tagged RLC; WT, wild type; MOPS, 4-morpholinepropanesulfonic acid; DTT, dithiothreitol; HMM/BP-RLC, HMM exchanged with benzophenone-4-iodoacetamide-labeled RLC (mutant not specified).

etamide; histRLC, His6-tagged RLC; WT, wild type; MOPS, 4-morpholinepropanesulfonic acid; DTT, dithiothreitol; HMM/BP-RLC, HMM exchanged with benzophenone-4-iodoacetamide-labeled RLC (mutant not specified).
Regulatory Light Chain Photocross-linking between Heads

Experimental Procedures

Protein Preparations—SMM was prepared from frozen chicken gizzards obtained from Pelt-Freeze. Smooth muscle HMM was obtained from Staphylococcus aureus protease (Sigma) digest of SMM (200–400 mg) as described previously (5) except a Superdex 200 gel filtration column was used (Amersham Pharmacia Biotech; 2.6 × 60 cm or 5.0 × 60 cm; 300 mM NaCl, 10 mM MOPS (pH 7.0), 0.1 mM EGTA, 1 mM MgCl2, 0.1 mM DTT, 0.5 mM dithiothreitol (Dithiothreitol)). Nucleotide gel analysis (24) showed that >95% pure HMM was obtained (densitometric scanning, Molecular Dynamics). SMM and HMM concentrations were determined using the following extinction coefficients: SMM, ε280 = 5.6; HMM, ε280 = 6.5. Truncated nonmuscle heavy meromyosin IIB, a brain isoform lacking both catalytic domains, was a gift from Dr. J. Sellers and Dr. F. Wang.

The light chain mutants were prepared by introducing a cysteine residue (introducing from) into the recombinant human smooth muscle RLC using oligo-directed mutagenesis and polymerase chain reaction techniques. Human smooth muscle RLC mutants Q15C, A23C, S59C, and T134C were prepared by substitution of Glu-15, Ala-23, Ser-59, and Thr-134 with cysteine, respectively, and Cys-108 of each was replaced by alanine. The DNA for the human RLC was subcloned into M13mp19 at the NdeI site at the end of the primer. The 3′-oligo sequence was 5′-GCCGAATTCGGCAAGGCCG-3′. The oligo for making the Cys-108 to alanine substitution was 5′-CGTCCGATCTGCGCAAGGCCG-3′. The oligo for A23C, S59C, and T134C mutants were antisense 5′-GGTCAACATCAACGAAGCATGTTG-3′, 5′-GCTTCTTCAGCCAGCAGCGCATG-3′, and 5′-CCCTGCTACATCGCCAAGGCGGTCAC-3′, respectively. Polymerase chain reaction using T7 polymerase was used to construct the mutant Q15C. The 5′-oligo introduced a NdeI restriction site at the start met-1 as well as introducing the Gln-15 to cysteine. The 5′-oligo sequence was 5′-CCGAGAAGCCT-ATGATGCTGACGGCCGCAAGAACGCCC-TCG-3′. The 3′-oligo used in polymerase chain reaction was complimentary to the 3′ end of the coding region of the RLC with an introduced EcoRI site at the end of the primer. The 3′-oligo was 5′-CCGGAAATTCCTACACCTGCGCCGC-3′. TA-cloning (Invitrogen) was done directly using the polymerase chain reaction mix with the Invitrogen protocol. Mutant RLC DNAs were confirmed by sequencing and then subcloned into the His-tag vector pET 15b (Novagen) adding 10 mM MgCl2, 0.1 mM DTT, 50 mM dithiothreitol (Dithiothreitol). Base-line separation was achieved between the exchanged HMM and the excess free RLC. HMM/BP-hisRLC fractions were pooled and precipitated with 2.5 volumes of saturated ammonium sulfate (pH 8.0) and then dialyzed to 15 mM Tris-Cl (pH 7.5), 150 mM KCl, 2.5 mM CaCl2, 10 mM MgCl2, 2 mM DTT. Thrombin (Sigma) was added (1 unit/mg HMM/BP-hisRLC), and the mixture was incubated on ice for 4 h. Densitometric scanning of 4–20% SDS-polyacrylamide gels showed that over 96% of His6 tagS were removed from the RLC by thrombin treatment. HMM/BP-RLC was dialyzed to 10 mM MOPS (pH 7.0), 0.1 mM EGTA, and 1 mM DTT immediately after thrombin treatment. HMM/BP-RLC was phosphorylated by incubating in 50 mM NaCl, 2.5 mM MgCl2, 2.5 mM CaCl2, 4 μg/ml calmodulin, 30 μg/ml myosin light chain kinase (27), and 1.5 mM ATP (Roche Molecular Biochemicals) at 25 °C and pH 7.0 for 1 h followed by incubating on ice overnight. Complete thio phosphorylation was verified by 8 μg/ml gel electrophoresis (4). Dephosphorylated control samples were prepared by adding 10 mM EGTA and 5 mM EDTA prior to adding the other components for thio phosphorylation. The buffer was changed to irradiation buffer (10 mM MOPS (pH 7.0), 0.1 mM EGTA, 1 mM DTT) by Sephadex G-50 (Sigma) centrifugal gel filtration (5-ml column, 0.8-ml load volume). HMM/BP-RLC solutions were centrifuged (350,000 × g) at 4 °C for 10 min, and the supernatant fractions were filtered through a 0.45-micron nylcon Acridine filter (Gelman). The two gel filtration steps, ultracentrifugation, and filtration were sufficient for removing aggregated HMM, as determined by native gel electrophoresis (24). Fresh DTT was added to 1 ml to the HMM/BP-RLC solutions before irradiation. To effect cross-linking, 200 μl of 1.2–1.4 mg/ml HMM solution in a 1.5-ml clear microfuge tube was irradiated through a Pyrex filter with a UV lamp (450 Watts, ACE Glass Incorporated) on ice as described previously (25).

Gel Electrophoresis and Western Blots—Gel electrophoresis was performed in the presence of 0.1% SDS with a 4–20% acrylamide gradient gel (Tris-glycine from Novex, San Diego, CA). Gels were stained with Coomassie Brilliant Blue. Sister gels were transferred to nitrocellulose for subsequent blotting. Western blots were performed by standard procedure (28) for both RLC and ELC antibodies. Polyclonal rabbit anti-RLC antibody and donkey anti-rabbit antibody labeled with horseradish peroxidase (Amersham Pharmacia Biotech) were used as the first and second antibodies, respectively, for RLC blotting. Mouse anti-ELC monoclonal antibody (a gift from Dr. K. Trybus) and sheep antimyosin antibody labeled with horseradish peroxidase (Amersham Pharmacia Biotech) were used as the first and second antibodies for ELC blotting. A Renaissance chemiluminescence kit (NEN Life Science Products) was used to visualize the antibody cross-reaction with RLC and ELC. Gels and films were scanned with a laser densitometer (Molecular Dynamics) for presentation.

Results

Fig. 1 documents a representative HMM preparation used in this study. HMM (Fig. 1, lane 2) was prepared from chicken gizzard myosin (Fig. 1, lane 1). Labeled mutant RLC (Fig. 1,
Regulatory Light Chain Photocross-linking between Heads

MgATPase activities of HMM and HMM/BP-RLC mutants were determined at 25 °C in 10 mM MOPS (pH 7.0), 0.1 mM EGTA, 1 mM MgCl₂, 1 mM DTT, 1 mM ATP at 0 and 25 μm actin. The rate of hydrolysis of [γ-³²P]ATP (DuPont) was measured (42). The time points for dephosphorylated HMMs (0.4 mg/ml; 2.1 μm heads) were quenched at 6, 12, 18, and 24 min. Thio-phosphorylated HMMs (0.2 mg/ml) were quenched at 2, 4, 6, and 8 min.

| HMM sample | Without actin | With actin |
|------------|---------------|------------|
|            | Dephos untreated | Dephos control | Thiophos |
| Control    | 2             | 2           | 1           | 4 | 186 |
| Q15C       | 1             | 3           | 5           | 6 | 332 |
| A23C       | <1            | 1           | <1          | <1 | 141 |
| WT         | 3             | 3           | 2           | 3 | 156 |
| S59C       | 4             | 4           | 1           | 1 | 121 |
| T134C      | 2             | 1           | 1           | 4 | 256 |

*The control was untreated HMM. The remaining samples were exchanged with the indicated RLC mutant labeled with BPIA (unirradiated).

* Values are averages of duplicate measurements. The range of the duplicates was within 2 nmol min⁻¹ mg⁻¹.

**TABLE I**

| HMM sample | Without actin |
|------------|--------------|
|            |              |
|            |              |

The effects of thio-phosphorylation upon the actin-activated ATPase activity of control HMM and all HMM/BP-RLC mutants (unirradiated) are shown in Table I. All samples behaved similarly to the control (unexchanged) HMM. As expected from previous studies (5), the HMMs lacked activation by thio-phosphorylation in the absence of actin but in the presence of actin showed an increase in activity upon thio-phosphorylation. The exact fold activation in the presence of actin because of thio-phosphorylation was difficult to quantify from these data because the dephosphorylated activities were low and close to our range of error. However, the activities of the thio-phosphorylated samples were all within a factor of 2 of the control. These results show that the HMM/BP-RLC preparations were regulated by thio-phosphorylation and therefore were used for subsequent irradiation studies.

Fig. 2 shows the effect of irradiation of HMM/BP-Q15C with UV light to activate the benzophenone moiety for cross-linking to proximal residues. In the dephosphorylated HMM (Fig. 2A, lane 1), irradiation generated a new band (lanes 2–5) migrating between 34.1 and 51.6 kDa (molecular mass markers not shown) and migrating just below actin (42 kDa) consistent with the expected behavior of a RLC-RLC dimer (RLC = 20 kDa). This band was identified as such after Western blot analysis showed cross-reaction with RLC (Fig. 2B) but not ELC (Fig. 2C) antibodies. The RLC-RLC dimer band was also generated in the presence of MgADP and MgATP (Fig. 2, lanes 8 and 9, respectively) and in the presence of actin (Fig. 2, lanes 10). The amount of RLC converted to the RLC-RLC dimer was estimated by scanning Coomassie-stained gels in the light chain region using the ELC for normalization of loaded protein. This analysis showed that the cross-linking efficiency was about 12% for all irradiation conditions, including in the presence of 125 and 500 mM NaCl (lanes 6 and 7, respectively). The RLC-RLC dimer band was the only new band observed after irradiation (Fig. 2). Cross-linking was not observed from the RLC to the heavy chain nor to the ELC. In addition intramolecular RLC cross-linking was not detected. To test for this, dilute solutions of free RLC were irradiated to determine the positions on the gels of intramolecularly cross-linked RLC. All new bands migrated below the unirradiated RLC (data not shown). However, these bands were not evident in experiments with the RLC bound to the heavy chain in the HMM (Fig. 2). Therefore, we concluded that the HMM-bound RLC did not cross-link intramolecularly but only intermolecularly to form RLC-RLC dimers.

In contrast to the results for the dephosphorylated HMM/ BP-Q15C, the identical experiment for the thio-phosphorylated protein showed no evidence for a RLC-RLC dimer (Fig. 2, D–F), except after irradiation in the presence of actin (Fig. 2, D and E). Therefore, the results for the HMM/BP-Q15C suggest that there is a clear conformational difference between the dephosphorylated and thio-phosphorylated states. The dephosphorylated state apparently positions Cys-15 (Gln-15 in the native RLC) from one head close enough to the other head to allow for cross-linking, whereas this event cannot occur in the thio-phosphorylated state.

As evidenced by RLC Western blots, this same general pattern of cross-linking was observed for the HMM/BP-A23C (Fig. 3, A and C) and for the HMM/BP-WT (Cys-108; Fig. 3, B and D). Cross-reaction was not observed with the ELC antibody under any condition (data not shown). Interestingly, these two mutants (both dephosphorylated and thio-phosphorylated), like Q15C, also showed generation of an RLC-RLC dimer after irradiation in the presence of actin.

The two other mutants studied, HMM/BP-S59C and HMM/ BP-T134C, showed a different behavior to those discussed above (Q15C, A23C, and WT (Cys-108)). In the dephosphorylated state no cross-linking was observed by Coomassie-stained
gels (data not shown) or Western blot analysis using RLC antibodies (Fig. 4, A and B), except for S59C in the presence of actin. As before, ELC cross-reaction was not observed under any condition (data not shown). Thiophosphorylated mutants (Fig. 4, C and D) also showed no cross-linking, and even the cross-linking observed for dephosphorylated S59C in the presence of actin was abolished. Therefore, except in the presence of actin for S59C, these mutants did not report a difference between the dephosphorylated and thiophosphorylated states.

Could the conformational differences between dephosphorylated and thiophosphorylated states that were detected for Q15C, A23C, and WT (Cys-108) be detected for a molecule without catalytic domains? To answer this question BP-Q15C-RLC was exchanged onto a truncated HMM-IIB (nonmuscle brain isoform) that lacked both catalytic domains but contained the full RLC and ELC binding sites and both the RLC and ELC. As found for the full-length HMM/BP-Q15C, irradiation of the truncated HMM generated RLC-RLC dimers in the dephosphorylated state (Fig. 5, lanes 2–4), whereas RLC-RLC dimers were barely detected in the thiophosphorylated state (Fig. 5, lanes 5–7), even with three times more protein loaded per lane. Western blot analysis with ELC antibodies (data not shown) showed no evidence for ELC-RLC cross-linking. Therefore the truncated HMM/BP-Q15C behaved like the full-length HMM/BP-Q15C with respect to the sensitivity of photocross-linking to the phosphorylation state, suggesting that the conformational change detected does not require the catalytic domains.

DISCUSSION

Table II summarizes the results from this study. We chose these conservative mutations as likely surface residues based upon an alignment with the chicken skeletal RLC sequence and its known three-dimensional structure (21). They also appear as surface residues in our homology model with the scallop regulatory domain (Fig. 6). Residues involved with the metal binding site were avoided because they would likely alter binding affinity of the RLC for the heavy chain. All of the mutants tested were found to exchange to similar levels and to be well regulated by thiophosphorylation (Table I), suggesting that the structural characteristics of the mutant RLCs were similar to the native protein.

3 C. R. Cremo, F. Wang, and J. R. Sellers, manuscript in preparation.
The labeled RLC mutants tested appeared to fall into two classes. In the first class of mutants, HMMs exchanged with the labeled mutants Q15C, A23C and WT (Cys-108) formed RLC-RLC dimers after irradiation but only in the dephosphorylated state. Upon thiophosphorylation, cross-linking was either not evident (Q15C and A23C) or substantially diminished (WT). The maximum length of the cross-linker from the cysteine sulfur to the photoreactive carbonyl carbon is about 8.9 Å. Therefore we conclude that the dephosphorylated state allows for these regions of the RLC to approach the RLC of the other head to within this distance. The approximate width of the RLC domain is about 25 Å. Our interpretation of the lack of cross-linking in the thiophosphorylated state is that these regions of the RLC are moving away from one another. We do not favor the alternative explanation that thiophosphorylation changes the positioning of selectively reactive amino acids, because of the relatively nonselective behavior of the benzophenone cross-linker used here (30) and because the same general behavior was observed for three mutants in this class. Our results are not consistent with the idea that the phosphorylated state has complete flexibility about the head-tail junction. This is because photocross-linkers may "trap" rare states with short lifetimes. If this mechanism were predominant, we would have expected to detect some cross-linking in the thiophosphorylated state for all mutant positions (also see mutants that show no cross-linking in either state).

It is significant that we observed phosphorylation-dependent cross-linking at increased ionic strength and in the absence of ATP (Figs. 2–4). This would argue that cross-linking was not correlated to a "10 S-like" state because the 10 S state is not stable at high ionic strength and also requires the presence of ATP (25). ATP and ADP did not strongly influence the RLC-RLC cross-linking efficiency. Our photocross-linking efficiency was most strongly influenced by thiophosphorylation and actin binding.

The Q15C mutant was also exchanged into a truncated non-muscle HMM IIB lacking both catalytic domains. As found for the native full-length HMM, RLC-RLC cross-linking was observed in the dephosphorylated state exclusively, suggesting that the catalytic domain is not necessary for the molecule to adopt the close positioning between the RLC domains. This result is generally consistent with the lack of effect of nucleotides upon our cross-linking results. Our findings also support fluorescence anisotropy measurements on a similar construct showing an increase in mobility of the RLC domain upon phosphorylation (31). Therefore, the catalytic domains are not required for phosphorylation-dependent changes in positioning between the two RLC domains.

In the second class of mutants, S59C and T134C, cross-linking was not observed in either the dephosphorylated or thiophosphorylated states (Fig. 4). Therefore we conclude that these regions of the RLC are faced away from the interfaces of the RLC that appear to approach closely as defined by the first class of mutants.

Ser-59 and Thr-134 (Class II) are located in the B-helix and at the end of the G-helix, respectively (Fig. 6A). Interestingly, these two residues are on opposite faces of the RLC from the residues in Class I (Fig. 6A). Before this study, little information had been available about the positions of the NH2-terminal region of the RLC, including Gln-15 and Ala-23, flanking the thiophosphorylated residue Ser-19, because the homologous residues from the skeletal myosin S1 (21) and the scallop regulatory domain (22) were found to be disordered. The equivalent region of the smooth muscle RLC is 24 residues in length. From our results with the Q15C and A23C mutants, it is clear that at least one of the interactions of this 24-residue region is
in close approach to the other head. Fig. 6B shows the relative positions of Phe-25 (the first visualized NH₂-terminal residue) and Cys-108, which is located at the top of the E-helix within the COOH-terminal domain of the RLC as defined by the break between the D and E Helix (21). It is interesting that Phe-25, which must be close to A23C, and Cys-108 are on the same face of the RLC and that both have the same cross-linking behavior (Class I).

An energy-minimized model of the head-tail junction with two scallop myosin regulatory domains (22) coupled to a model of the scallop tail, shows specific interactions between the two RLC (32) in a pseudo-symmetrical relationship. This model shows Gln-46 (scallop; equivalent to our S59C mutant) close to three residues in the COOH-terminal domain of the RLC of the other head. In our study S59C shows no RLC-RLC cross-linking, suggesting that at least in this myosin, the contact is not within 8.9 Å. The scallop model also does not predict the cross-linking we observe from Cys-108. Therefore in general our results are not in accordance with the scallop model.

| Mutant RLC on HMM | Cross-linking in absence of actin | Cross-linking in presence of actin |
|-------------------|----------------------------------|----------------------------------|
|                   | dephos | thiophos | dephos | thiophos |
| Q15C              | +      | -       | +      | ND      |
| Q15C (no catalytic domains) | -      | -       | ND     | ND      |
| A23C              | +      | +       | +      | +       |
| WT (Cys-108)      | +      | +       | +      | +       |
| S59C              | -      | -       | +      | +       |
| T134C             | -      | -       | -      | -       |

Table II: Summary of RLC-RLC photocrosslinking

A plus sign designates that an RLC-RLC cross-link was detected, whereas a minus sign indicates either no cross-link was detected or a significant decrease in cross-linking was observed. ND, not determined.
HMM complex, the head-head separation is diminished. This result seems counterintuitive. Binding of HMM heads to adjacent actin monomers would be expected under these conditions. Using the skeletal actoS1 structure as a model (21), two heads attached to adjacent monomers would most certainly involve some distortion in the coiled-coil tail to allow for separation of the RLC domains. Our results suggest that for smooth muscle HMM, the conformations of the heads when bound to actin must differ from this model in the region of the RLC, allowing for proximity of the two RLCs. In contrast, the acto S1 model (21) is consistent with cross-linking between the catalytic domains observed in the smooth actoHMM complex (33). Further experiments are required to establish the molecular basis for our findings.

It has been proposed that full regulation of SMM requires interaction of the heads with the S2 portion of the tail (20) in the dephosphorylated state. However, results from deletion mutants of the S2 portion of the rod suggest that a specific amino acid sequence at the head-rod junction may not be critical for full regulation (34). It is interesting to note that, at least for the mutants tested, we did not observe RLC-heavy chain cross-linking, suggesting that the mutant residues did not approach the heavy chain within 8.9 Å. Atomic force images of smooth muscle myosin also did not show clear evidence for interaction of the RLC with the tail region (37).

It is interesting to place our results into the context of previous studies of the effects of phosphorylation on the structure of regulated HMMs. It is difficult to compare results with whole myosin because of its ability to adopt the filamentous and folded (10 S) forms that are not available to the HMM fragment. To date, phosphorylation of smooth muscle HMM has been correlated to four effects. Phosphorylation increases the sedimentation coefficient (35), stabilizes a conformation that appears visually to have the heads extended away from the tails (in contrast to heads bent back toward the tail) (35), increases proteolytic susceptibility of the head-tail junction (36), and increases the mobility of the active site region and the RLC (31). A cryo-atomic force microscopy study showed that the range of separation between the two heads was increased by thiophosphorylation of 6 S smooth muscle myosin molecules at high ionic strength (37). Together these studies show an immobilized dephosphorylated head-tail junction, with closer heads, and a more compact overall structure than the phosphorylated state. Studies with scallop HMM (calcium-regulated) show similar trends induced by calcium binding (38). However, from these relatively low resolution studies it is not possible to compare detailed molecular interactions controlled by phosphorylation.

Our study provides novel information through the use of a site-directed chemical technique that provides higher resolution information about many sites within the RLC subunit. The labeled mutants report a conformation specific to dephosphorylated smooth HMM that involves a close juxtaposition of the two RLC subunits and a separation of the RLC subunits upon phosphorylation. Class I mutations appear to be at the interface between the two RLCs (approaching within 8.9 Å). Our method does not distinguish between a close-fit interaction (tight) and one that is weak but within distance range of the photocross-linking reagent. We found that Class II mutations are unable to cross-link in HMM, which is expected behavior for probes that do not have access to the interface between the RLCs. We are currently extending the power of the photocross-linking technique to map the sites of cross-linking and to further examine the positioning of the RLC in actoHMM conformations, which are difficult to study with other methods.

Acknowledgments—We thank Geetha Ramapriyan for protein preparations, XiaFeng Li for assistance with some of the light chain mutants, and Sam Mazhari and Dr. Jeff Jones for the homology model. We thank Dr. G. Offer for sharing the coordinates of the model of the head-tail junction.

REFERENCES
1. Harthorne, D. J. (1987) Biochemistry of the Contractile Process in Smooth Muscle: Physiology of the Gastrointestinal Tract, 2nd Ed., (Johnson, L. R., ed) pp. 425–481, Raven Press, New York
2. Sellers, J. R. (1991) Curr. Opin. Cell Biol. 3, 98–104
3. Sellers, J. R., and Goodman, H. (1995) Protein Profile 2, 1233–1423
4. Crema, C. R., Sellers, J. R., and Facemeyer, K. C. (1995) J. Biol. Chem. 270, 2171–2175
5. Ikebe, M., and Harthorne, D. J. (1985) Biochemistry 24, 2380–2387
6. Seidel, J. C. (1980) Biochemistry 19, 4412–4419
7. Ikebe, M., and Hartshorne, D. J. (1985) Biochemistry 24, 2380–2387
8. Matsuura, M., and Ikebe, M. (1995) FEBS Lett. 363, 246–250
9. Sata, M., Matsuura, M., and Ikebe, M. (1996) Biochemistry 35, 11113–11118
10. Trybus, K. M., Freyzo, Y., Fava, L. Z., and Sweeney, H. L. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 48–52
11. Flicker, P. F., Wallimann, T., and Vibert, P. (1983) J. Mol. Biol. 169, 723–741
12. Ikebe, M., Reardon, S., Mitani, Y., Kaminosyama, H., Matsuura, M., and Ikebe, R. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9096–9100
13. Rowe, T., and Kendrick-Jones, J. (1993) EMBO J. 12, 4877–4884
14. Ikebe, M., Kambara, T., Stafford, W. F., Sata, M., Katayama, E., and Ikebe, R. (1998) J. Biol. Chem. 273, 17792–17797
15. Ikebe, M., Ikebe, R., Kaminosyama, H., Reardon, S., Schwonek, J. P., Sanders, C. R., II, and Matsuura, M. (1994) J. Biol. Chem. 269, 28173–28180
16. Trybus, K. M., and Chatman, T. A. (1993) J. Biol. Chem. 268, 4412–4419
Regulatory Light Chain Photocross-linking between Heads

17. Trybus, K. M. (1994) J. Biol. Chem. 269, 20819–20822
18. Rovner, A. S., Freyzon, Y., and Trybus, K. M. (1995) J. Biol. Chem. 270, 20819–20822
19. Sata, M., Stafford, W. F., Mabuchi, K., and Ikebe, M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 91–96
20. Trybus, K. M., Naroditskaya, V., and Sweeney, H. L. (1998) J. Biol. Chem. 273, 30260–30263
21. Xie, X., Harrison, D. H., Schlichting, I., Sweet, R. M., Kalabokis, V. N., Szent-Gyorgyi, A. G., and Cohen, C. (1994) Science 268, 306–312
22. Ikebe, M., and Hartshorne, D. J. (1985) J. Biol. Chem. 260, 13146–13153
23. Persechini, A., Kamm, K. E., and Stull, J. T. (1986) J. Biol. Chem. 261, 6293–6299
24. Odrey, J. J., Sellers, J. R., and Creme, C. R. (1996) J. Biol. Chem. 271, 25372–25384
25. Facemyer, K. C., and Creme, C. R. (1992) Bioconjugate Chem. 3, 408–413
26. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., sec. 18.60–18.72, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
27. Ikebe, M., Yamada, M., Mabuchi, K., Kambara, T., and Ikebe, R. (1998) Biochemistry 37, 13285–13290
28. Ikebe, M., and Hartshorne, D. J. (1984) J. Biol. Chem. 259, 11639–11642
29. Zhang, Y. Y., Shao, Z. F., Somlyo, A. P., and Somlyo, A. V. (1997) Biophys. J. 72, 1308–1318
30. Frado, L. L. Y., and Craig, R. (1992) J. Muscle Res. Cell Motil. 13
31. Cole, D. G., and Yeung, R. G. (1996) J. Biol. Chem. 271, 22537–22546
32. Offer, G., and Knight, P. (1990) J. Mol. Biol. 256, 407–416
33. Onishi, H., Maita, T., Matsuda, G., and Fujiwara, K. (1990) J. Biol. Chem. 265, 19362–19368
34. Ikebe, M., and Hartshorne, D. J. (1985) J. Biol. Chem. 260, 13146–13153