Chimeric substitution of the weak actin-binding loop (ABL) from chicken skeletal muscle myosin for that of gizzard smooth muscle heavy meromyosin (HMM) causes activation of the dephosphorylated mutant (SABL HMM; Rovner, A. S., Freyzon, Y., and Trybus, K. M. (1995) J. Biol. Chem. 270, 30260–30263). The present study determined whether this loss of regulation is due to the greater positive charge density (5 versus 3 clustered lysine residues) or lesser length (14 versus 26 residues) of the mutant ABL. Charge augmentation had little effect on regulation of expressed mutants, but elimination of the 12 N-terminal amino acids from the wild-type ABL significantly increased actin-activated ATPase activity of the dephosphorylated relative to the phosphorylated molecule while conferring the ability to move actin filaments in vitro on the former. Addition of the same 12 residues to the SABL mutant increased the ratio of phosphorylated to dephosphorylated ATPase activity while imparting wild type-like regulation to motility. However, full actin activation of dephosphorylated ATPase activity required both the shorter length and greater positive charge density found in the SABL loop. These results demonstrate that, compared with skeletal, both the greater length and lesser positive charge density of the smooth muscle myosin ABL are required for proper phosphorylation-mediated regulation of the molecule.

Unlike the myosins from striated muscle, vertebrate smooth muscle myosin requires phosphorylation of the regulatory light chain (RLC) to activate its enzymatic and mechanical activities (reviewed in Ref. 1). Early theories suggested that activation of the molecule is caused by a change in shape that occurs in response to RLC phosphorylation (2, 3), but this idea was refuted by separating its effects on molecular folding and activity (4). It is now widely accepted that phosphorylation activates the molecule by accelerating the rate-limiting step of the acto-myosin ATPase cycle independent of any gross changes in shape (5).

Since the discovery of this “thick filament-linked” form of regulation, there have been intensive efforts to determine the mechanism whereby RLC phosphorylation in the neck region of myosin causes activation of the 80-angstrom distant nucleotide-binding cleft. Experiments with chimeric RLCs suggested that the regulatory signal is propagated to the active site by physical overlap between the RLC and the essential light chain (6). However, a hybrid smooth muscle HMM containing the smooth RLC and skeletal essential light chain was well regulated in the actin filament motility assay, as was an essential light-chain-less molecule (7), refuting the role of the latter subunit. Other studies focusing on the RLC showed that complete activation of smooth muscle myosin requires specific arrangements of charge both at the N terminus (8, 9) and C terminus of the molecule (10). However, the manner in which these charged groups contribute to activation remains unknown.

Additional important information shows that proper regulation requires a two-headed structure. The enzymatic activity of the single-headed subfragment-1 (S1) was fully activated by actin in the absence of RLC phosphorylation (11, 12), which was also essentially true for HMM molecules with a single head (13). An expressed short HMM with a 27-nm-long tail existed in an equilibrium between single- and double-headed species and was much more poorly regulated than a 72-nm tail-length long HMM, which was uniformly double-headed (7). More recent studies indicate that the attributes of the long α-helical 20-kDa sequence comprising the “neck” of each cross-bridge (14), as well as the sequence C-terminal to where the two heads join (the subfragment-2 or S2 portion) are instrumental in determining whether or not a given molecule is tightly regulated (15). These results indicate that some interaction between the two cross-bridge heads is crucial to the ability of the molecule to be held in the “off” state in the absence of RLC phosphorylation.

Recently, we showed that chimeric substitution of chicken pectoralis myosin sequence for the endogenous chicken gizzard sequence at the site of the flexible surface loop connecting the 50- and 20-kDa tryptic domains of smooth muscle HMM (the actin-binding loop or ABL) caused actin activation of the dephosphorylated molecule (16). Comparison of the primary sequences of the wild-type smooth muscle ABL and the chimera with skeletal sequence substituted (named SABL HMM) highlights two major attributes that may be responsible for the difference in their regulatory behavior (Fig. 1A). First, the SABL mutant has five rather than three positive charges clustered in the lysine pocket at the C-terminal end of the loop. Second, the smooth muscle ABL is 12 amino acid residues longer than the skeletal loop. These specific differences in charge density and length are always maintained when the ABL sequences from regulated vertebrate myosin II isoforms are compared with sequences from non-regulated myosins, sug-
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suggesting that either one or both of these attributes may be related to the ability of a myosin to be regulated by RLC phosphorylation.

Given these differences, the goal of the present study was to separately test the importance of ABL positive charge density and loop length in the ability of smooth muscle myosin to stay “turned off” in the absence of RLC phosphorylation. To this end, the baculovirus system was used to express mutant smooth muscle HMM molecules with increased ABL positive charge, decreased ABL length, or both, and the enzymatic and motile activity of these molecules was compared in the phosphorylated and dephosphorylated states. The results indicate that optimal regulation requires both decreased positive charge density in the lysine pocket and a longer loop and underscore the complexity of interactions involved in the regulation of activity in smooth muscle myosin.

EXPERIMENTAL PROCEDURES

Construction of Chimeric Mutants of the Actin-binding Loop—The chicken gizzard myosin heavy chain cDNA (17) was used as the base template for all constructs used in this study. Deletions or additions of sequence to the ABL were performed using four primers in a polymerase chain reaction technique (PCR), which has been well described (18). In the case of the ABL-12 mutant, in which the first 12 amino acids of the smooth muscle ABL were removed, the two oligonucleotides used to introduce the deletion had the sequence TTTCCATCATCTGTCGGC-CAAAAGGCTGTCGCCAACAAAT for the antisense primer of the more 5′ PCR product and TGGTGCAGACCTTTCGGCCAAATGATGACTGAGACGTT-GTCC for the sense primer of the 3′ PCR product. The effect of these mutations was to juxtapose the codons for Tryptophan 625 and Alanine 638 of the smooth muscle myosin heavy chain sequence. For the SABL+12 mutant, in which the same 12 amino acids were added upstream of the skeletal ABL sequence in the SABL mutant, the oligonucleotides used were CTAAAGCTCATTTCTGGGTCGACCATCTTGCCGAAGGTGTGGC-CAAAATGATGACTGAGACGTT-GTCC for the antisense primer of the more 5′ PCR product and CGA-GGCTCTTCTGGGTCGACCATCTTGCCGAAGGTGTGGC-CAAAATGATGACTGAGACGTT-GTCC for the sense primer of the 3′ PCR product. These manipulations introduced the amino acid sequence KDVRIVGLDQM prior to Glycine 626 of the sequence of the SABL mutant (see Fig. 1C). The SABL mutant was synthesized as described previously (16). To create the KABL, KKABL, and KRKABL-12 mutants, site-directed mutagenesis was carried out using the Altered Sites kit (Promega). The accuracy of all mutated sequences was verified by dye-deoxy sequencing.

Production of Recombinant Baculoviruses and HMM Expression in SF9 Cells—All ABL mutations were produced in a fragment of the chicken gizzard heavy chain cDNA (17), which was cloned into the baculovirus transfer plasmid pVL1392 to produce an HMM fragment of 1175 amino acids (16). Recombinant baculoviruses were produced by simulating the culture and transfection of SF9 cells with these transfer plasmid constructs and engineered baculoviral DNA (Baculogold; Pharmingen). Myosin heavy chain viral stocks were amplified by established procedures (19) and then used to co-infect SF9 cells along with a virus driving expression of both the essential light chain and the RLC from the sense primer of the 3′ PCR product. This created a spacial distribution of positive charge over the C-terminal portion of the ABL alone is responsible for the activation of dephosphorylated smooth muscle myosin. HMM molecules with alterations in either ABL positive charge distribution or length were expressed as described under “Experimental Procedures,” and their regulatory behavior was examined by comparing the enzymatic and motile activity of phosphorylated and dephosphorylated preparations.

Increased Positive Charge Density in the Smooth Myosin ABL Does Not Greatly Activate the Dephosphorylated Molecule—Initial experiments were conducted to determine whether additional positive charge at the C-terminal end of the ABL alone is responsible for the activation of dephosphorylated SABL relative to the wild-type molecule. In the KABL mutant, Alanine 648 was replaced by a lysine residue, whereas in the KKABL mutant, both Serine 647 and Alanine 648 were replaced with lysines (Fig. 1B). This created a spacial distribution of positive charge over the C-terminal portion of the smooth ABL in the latter essentially identical to that in the SABL mutant (Fig. 1A).

Fig. 2 panels A–C compare the actin-activated ATPase activity of these constructs with that of the wild-type molecule. The addition of positive charge to the lysine residue of the ABL caused a progressive increase in $V_{\text{max}}$ from about 3.9 s⁻¹ for the phosphorylated wild-type HMM (panel A) to about 8 s⁻¹ in the dephosphorylated KKABL mutant (panel C). In the case of the dephosphorylated molecules, the augmentation of positive charge also increased activity compared with dephosphorylated wild type. Nonetheless, the activity of the phosphorylated mutants was severalfold greater than that of their dephosphorylated counterparts. The degree of regulation, calculated as the
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The experiments described above showed that the average velocity of actin filament motility of expressed wild-type smooth muscle myosin ABL, created an ABL the same length as that of the SABL mutant. In this case, the ABL was not responsible for activation of the dephosphorylated SABL HMM mutant (16). It was therefore important to determine whether the activation is due only to the activity of the phosphorylated molecule at 80 μM actin, whereas the value for dephosphorylated ABL-12 was also in this range. These changes in activity decreased the degree of regulation from over 9.3 in the wild-type molecule to 1.8 in ABL-12 (see Table I). Conversely, addition of the 12 amino acids to the N terminus of the SABL loop caused a significant decrease in the velocity of actin filament motility of expressed wild-type smooth muscle myosin ABL (reprinted from Fig. 2). C. SABL HMM mutant; D, SABL+12 HMM mutant. Curves were derived from least squares fits of the data points as described in the legend to Fig. 2.

**TABLE I**

Phosphorylation-dependent regulation of expressed smooth muscle HMMs

| Preparation | Actin-activated ATPase s⁻¹ | Degree of regulation |
|-------------|---------------------------|----------------------|
| Wild type   | 2.05                      | 9.3                  |
| KABL        | 2.98                      | 3.9                  |
| KKABL       | 4.40                      | 5.1                  |
| ABL-12      | 5.04                      | 1.8                  |
| SABL        | 3.56                      | 1.15                 |
| SABL + 12   | 2.21                      | 2.5                  |
| KKABL − 12  | 3.94                      | 1.2                  |

The velocity of actin filament motion supported by the two mutant HMM molecules is presented in Fig. 2D. The addition of positive charge caused a decrease in velocity from a value of 0.7 μm/s in the phosphorylated wild-type molecule to 0.55 μm/s for KABL in the phosphorylated state. In the dephosphorylated state, none of the three molecules showed any discernible movement, further supporting the contention that the addition of positive charge to the ABL does not significantly alter the regulatory properties of smooth muscle HMM.

Twelve Amino Acids at the N Terminus of the Smooth Muscle Myosin ABL Inhibit Actin Activation of ATPase Activity and Motility—The experiments described above showed that the increased positive charge density at the C-terminal end of the ABL was not responsible for activation of the dephosphorylated SABL HMM mutant (16). It was therefore important to determine whether the activation is due only to the significantly shorter length of the SABL loop. To this end, the ABL-12 and SABL+12 mutants illustrated in Fig. 1C were synthesized using the PCR technique described under "Experimental Procedures." In ABL-12, 12 amino acids were removed from the N terminus of the wild-type smooth muscle myosin ABL, creating a loop that is the same length as that of the SABL mutant. In the SABL+12 mutant, the reverse approach was taken by adding the same peptide of 12 amino acids onto the N terminus of the SABL loop sequence, creating an ABL the same length as the wild-type smooth muscle molecule.

In Fig. 3, the actin-activated ATPase activities of these two mutants are compared with the activity of the wild-type expressed molecule and the SABL mutant. Subtraction of the 12 residues from the wild-type molecule to create ABL-12 caused a marked increase in activity both of the phosphorylated and dephosphorylated molecules (compare panel B with panel A). The calculated Vmax for phosphorylated ABL-12 was nearly 6 s⁻¹ compared with 3.9 s⁻¹ for phosphorylated wild-type, whereas the value for dephosphorylated ABL-12 was also in this range. These changes in activity decreased the degree of regulation from over 9.3 in the wild-type molecule to 1.8 in ABL-12 (see Table I). Conversely, addition of the 12 amino acids to the N terminus of the SABL loop caused a significant decrease in the activity of the dephosphorylated molecule, from over 5 s⁻¹ in SABL to less than 2 s⁻¹ in SABL+12 (compare panel D with panel C). Vmax in the phosphorylated state was essentially unchanged at 4.2–4.4 s⁻¹. Thus, the SABL+12 molecule was significantly more tightly regulated than SABL, with a degree of regulation of 2.5 rather than 1.15 (Table I).

The changes in length of the loop also had a profound impact on the value of KATPase, particularly in the phosphorylated state. Phosphorylated ABL-12 had a KATPase of ~10 μM compared with a figure closer to 65 μM for the wild-type molecule, whereas the KATPase for SABL+12 was nearly four times greater than that of the SABL mutant. This indicates that the additional residues at the N terminus of the smooth muscle ABL may interfere with the interaction of the dephosphorylated smooth muscle cross-bridge with actin and that this additional sequence may be required in smooth muscle myosin to support any discernible movement.
help keep the molecule “turned off.”

Addition or subtraction of the 12 amino acids had the same qualitative and quantitative effects on unloaded actin filament motility as on ATPase activity (Fig. 4). Removal of the 12 N-terminal residues from the ABL caused activation of dephosphorylated ABL-12, which moved actin filaments at a velocity ~50% as great as that of phosphorylated ABL-12. As previously demonstrated, dephosphorylated SABL maintained at least the same velocity as its phosphorylated counterpart (16). However, addition of the N-terminal sequence to SABL created a fully regulated SABL+12 molecule that was incapable of movement in the absence of RLC phosphorylation. Notably, removal of the 12 amino acids caused a marked decrement in unloaded velocity for both phosphorylated and dephosphorylated ABL-12 and SABL. However, addition of these residues to the N terminus of the ABL restored the velocity of phosphorylated SABL+12 to wild-type levels.

Both Greater Length and Lesser Positive Charge Density of the ABL Are Required for Full Regulation of Smooth Muscle HMM—The preceding results showed that neither increased positive charge density nor removal of the 12 N-terminal amino acids of the smooth muscle ABL is sufficient to account for the nearly complete actin activation of the ATPase of the dephosphorylated SABL mutant. It therefore seemed reasonable to postulate that the combination of these two factors is required. This was tested by the KKABL-12 mutant (Fig. 1D), in which the replacement of adjacent serine and alanine residues in ABL-12 with lysines created an HMM essentially identical to the SABL mutant from the generic standpoint of loop length and lysine charge density (compare with Fig. 1A). In the actin-activated ATPase assay, the regulatory behavior of KKABL-12 was strikingly similar to the SABL mutant (Fig. 5A), as the activity of the dephosphorylated molecule at every actin concentration was nearly as great as the phosphorylated activity. Removal of the 12 amino acids caused a marked decrement in the unloaded velocity of KKABL-12 compared with wild type HMM. Consistent with these findings, the presence here of two additional charges in the KKABL, SABL, and KKABL-12 mutants caused only a 20–100% increase in ATPase V\text{max} in the phosphorylated state compared with wild type. This suggests that 1) the simple addition of one or two positive charges to the lysine pocket of the ABL does not significantly impact upon myosin function and 2) the 5-fold effect on ATPase seen previously (21) depends upon other non-charged elements of the skeletal sequence and its interaction with the Dictyostelium backbone.

A Long ABL Sequence Is Required in Addition to Decreased Positive Charge Density for Tight Regulation of ATPase Activity—Removal of the 12 N-terminal amino acids from the smooth ABL had a profound effect on actin-activated ATPase, significantly stimulating the activity of the dephosphorylated molecule and decreasing the degree of regulation from 9.3 in the wild type to 1.8 in the ABL-12 mutant. The additional demonstration of an increase in the degree of regulation of

**FIG. 4.** Unloaded *in vitro* actin filament motility supported by expressed wild-type HMM (reprised from Fig. 2) and mutants in which the 12 N-terminal amino acids of the ABL were subtracted or added. Values presented are the mean of three preparations ± the standard deviation. Filled boxes are phosphorylated preparations, and open boxes are dephosphorylated preparations.
SABL+12 compared with SABL therefore suggests that the extra amino acid residues in the smooth muscle loop compared with the skeletal ABL play an “inhibitory” role and are important in “turning off” the activity of the dephosphorylated molecule. This proposal is further supported by the finding that dephosphorylated SABL+12 failed to move actin in the motility assay. However, the ATPase activity of dephosphorylated SABL+12 was still significantly greater than the dephosphorylated wild-type molecule (compare panels A and D in Fig. 3), refuting the idea that loop length alone is sufficient to determine the regulatory properties of wild-type smooth muscle myosin. The KKABL-12 mutant, possessing an ABL with the same generic length and charge characteristics of SABL, had a degree of regulation essentially no different than the latter skeletal-like molecule. Collectively, these findings show that the ability of wild-type smooth muscle HMM (and myosin) to be tightly regulated by RLC phosphorylation depends upon the presence of an ABL, with greater length and lesser positive charge density than the skeletal isofrom.

Is the mechanism by which the shorter, more charged ABL activates the molecule the same as RLC phosphorylation? Sellers et al. (23) showed that in the presence of ATP, phosphorylation of smooth HMM caused only a 4-fold increase in binding to actin (and a corresponding ∼4-fold decrease in $K_{\text{ATPase}}$) while increasing $V_{\text{max}}$ of ATPase 25-fold. Later work using non-steady-state techniques indicated that the latter increase may be more on the order of 1000-fold (5). Here, comparison of the ATPase curves for dephosphorylated SABL and KKABL-12 (Figs. 3C and 5A) with phosphorylated wild type (Fig. 3A) shows that modification of the loop has an effect very similar to phosphorylation of the wild-type molecule. The values of $V_{\text{max}}$ for the two dephosphorylated mutants are about 30% greater than that for the phosphorylated wild type (∼5.7 s⁻¹), whereas $K_{\text{ATPase}}$ is similar for all of these species (in the range from 55–95 μM actin). Comparison of these values with those published for dephosphorylated smooth HMM (0.071 s⁻¹ and 133 μM, respectively; Ref. 23) suggests that truncation and charge augmentation of the ABL activates the molecule by accelerating the same step in the cross-bridge cycle affected by RLC phosphorylation. This supposition is also consistent with direct binding measurements on the SABL mutant, which showed that its affinity for actin in the dephosphorylated state was no different than the wild-type molecule (16).

Despite the above similarities, RLC phosphorylation further alters the function of the ABL mutants, indicating clearly that the changes in loop sequence do not by themselves exactly emulate the kinetic effects produced by this covalent modification. Phosphorylation of SABL or KKABL-12 (as well as ABL-12) causes a 4–10-fold decrease in $K_{\text{ATPase}}$ with essentially no change in $V_{\text{max}}$. The $K_{\text{ATPase}}$ effect is consistent with the increase in $K_{\text{binding}}$ observed by the NIH group (23) but by no means proves that RLC phosphorylation increases actin binding, as $K_{\text{ATPase}}$ is influenced by more than one elementary rate constant in a model-dependent way (24). However, the fact that phosphorylation produces this extra effect at low actin concentrations in addition to the activation caused by changes in loop sequence strongly argues that the steps in the cross-bridge cycle affected are different. Further study of the impact of these loop modifications on the elementary reactions of the cross-bridge cycle using non-steady-state techniques should allow further insight into the mechanism by which smooth muscle myosin enzymatic activity is regulated.

Charge Augmentation and Truncation Decrease Actin Filament Motility—Interestingly, both charge augmentation and truncation of the loop caused a progressive decrease in the velocity of unloaded actin filament motility for phosphorylated mutant molecules relative to wild type. Thus, these alterations have quite different effects on the enzymatic and mechanical properties of the molecule, as was shown in the comparable Dictyostelium mutants (21). How can this uncoupling of function be explained? In the motility assay, unloaded velocity $V$ is dependent upon the length of each power stroke of the myosin molecule ($d_{\text{uni}}$), the ATPase rate, and the unloaded duty cycle ($f_{\text{unload}}$), which is the proportion of time in a single cross-bridge cycle during which myosin is strongly attached to and propelling the actin filament (25):

$$V = \frac{(d_{\text{uni}})(\text{ATPase rate})}{f_{\text{unload}}} \quad (\text{Eq. 1})$$

Based upon this expression, it is obvious that the decreased velocity in the charge alteration and loop truncation constructs must be due either to a decrease in $d_{\text{uni}}$ or an increase in $f_{\text{unload}}$, since the ATPase rate in each case has increased or stayed the same. Recent evidence suggests that $d_{\text{uni}}$ is a mechanical property that depends upon the length of the “neck” region of the cross-bridge, which may amplify small conformational changes arising in the motor domain by acting as a swinging lever arm (26, 27). Since the ABL mutations in question are situated at the opposite end of the molecule from the neck region, it seems unlikely that they would affect $d_{\text{uni}}$. Therefore, these mutants probably increase $f_{\text{unload}}$ by increasing the proportion of the cross-bridge cycle in which myosin is strongly bound to actin. The kinetic step thought to determine the duration of this attachment is ADP release from the active site pocket (28). Thus, one implication of these results is that greater charge density or lesser length in the ABL slows the rate of ADP release from the nucleotide pocket.

Relationship to Recent Models of Smooth Muscle Myosin Regulation—The work in this paper describes the importance of structural variation in the putative actin-binding interface in the determination of myosin’s regulatory properties. Two recent publications implicate the importance of other sites in this process. Sata et al. (14) used the baculovirus system to express a chimeric HMM with a skeletal myosin motor domain (amino acids 1–773) and a smooth myosin neck + S2 region (amino acids 774–1104). They showed that this molecule was regulated nearly as tightly as native smooth muscle HMM for both ATPase and motile activity, with ATPase $V_{\text{max}}$ nearly identical to that of biochemically prepared skeletal HMM. They therefore postulated that the structure of the neck region alone is sufficient to determine the regulatory properties of the molecule, whereas the motor domain is sufficient to determine its enzymatic activity. However, the motility of the chimeric construct was almost 5-fold slower than the velocity evidenced by their expressed wild-type smooth muscle HMM (only 0.12 μm/s), contrasting with the observation that chimeric molecules with the smooth myosin motor domain and skeletal myosin neck region support velocities comparable with the wild-type molecule. This suggests that in engineering the junction between the two domains in their chimera, Sata et al. (14) introduced a compliant element into the molecule that diminishes its performance. Additionally, their quantitative electron microscopic assay of the relative numbers of “folded” and “extended” chimeric molecules was not compared with a skeletal muscle HMM control. Therefore, it is not clear that the changes they saw in the shape of the chimeric molecule were strictly due to the presence of the smooth regulatory domain, and thus their work does not rule out the importance of other sites in the regulation of the molecule.

Trybus et al. (15) used the same expression system to pro-

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2 K. M. Trybus, personal communication.
duce a series of smooth muscle myosin head fragments, which were truncated at various points in the S2 region. They confirmed previous observations that a double-headed molecule is required for a reasonable level of regulation by RLC phosphorylation (7, 13, 29), substantiating the idea that this process somehow involves a direct interaction of the two heads. However, this group also showed that the tightest regulation requires the S2 sequence linking the heads to have a length at least equal to that of the heads themselves. This finding implies that full regulation requires interaction of a site on the heads far removed from the head-rod junction with a site on S2, thus stabilizing a specific geometric relationship of the two heads. The importance of structural variation in the ABL and its location at the farthest point of the motor domain from the head-rod junction suggests that it may at least in part mediate the interaction of the smooth muscle myosin heads either with the S2 sequence or with each other. These observations show that the regulation of activity in smooth muscle myosin involves a complex series of interactions between sites in the head and neck regions of the cross-bridge. Future success in our understanding of the regulatory process will hinge on our ability to elucidate the mechanism by which these different domains of the molecule interact, both in the presence and absence of RLC phosphorylation.

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Note Added in Proof—While this manuscript was under review, another report appeared demonstrating that smooth muscle sequence and charge affect smooth myosin regulation

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