Chimeric Substitutions of the Actin-binding Loop Activate Dephosphorylated Smooth Muscle Heavy Meromyosin*

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Regulatory light chain (RLC) phosphorylation is necessary to activate smooth muscle myosin, unlike constitutively active striated muscle myosins. Here we show that an actin-binding surface loop located at the 50/20-kDa interface of the myosin heavy chain; with that from either skeletal or β-cardiac myosin caused the chimeric HMMs to become unregulated like the myosin from which the loop was derived. Dephosphorylated chimeric HMMs gained the ability to move actin in a motility assay and had 50–70% of the actin-activated ATPase activity of phosphorylated wild-type HMM. Direct binding measurements showed that the affinity of HMM for actin in the presence of MgATP was unaffected by loop substitution; thus the rate of a step other than binding is increased. Phosphorylation of the chimeras did not lead to a higher V\text{max} than obtained for wild-type HMM. In the absence of actin, a foreign loop did not affect nucleotide trapping. Native regulated molecules have thus evolved a loop sequence which prevents rapid product release by actin when the RLC is dephosphorylated, thereby allowing activity to be controlled by RLC phosphorylation.

Myosins differ from each other in how fast they can move actin, the rates of their actin-activated ATPases, and whether or not these activities are regulated. It has been recently suggested that a divergent surface loop at the actin-binding interface "tunes" the rate of phosphate release and thus sets the maximum velocity (V\text{max}) for ATPase activity. A second loop, at the 25/50-kDa interface near the ATP-binding site, was proposed to control the rate of ADP release and thus be responsible for determining the velocity at which different myosins move actin (Spudich, 1994). The actin-activated ATPase activity of a chimeric Dictyostelium myosin containing the actin-binding loop from skeletal muscle myosin was 5-fold higher than wild-type Dictyostelium myosin, providing experimental evidence in support of the first part of this hypothesis (Uyeda et al., 1994). This study did not show if such a generalization would hold true for other myosin motors nor was the question of heavy chain sequences involved in regulation of activity addressed.

The molecular step that is controlled by light chain phosphorylation in smooth muscle myosin is phosphate release from the active site (Sellers, 1985). It has been recently proposed that the myosin is a "back door" enzyme, whereby the released phosphate leaves via a defect in the 50-kDa domain, instead of through the nucleotide-binding pocket from which it entered. Binding of actin is suggested to promote movement of the highly conserved P-loop near the active site so that phosphate can leave (Yount et al., 1995). The interaction of actin with myosin probably involves several steps; the first is thought to be a weak interaction between the N terminus of actin and the 50/20-kDa junction of myosin, followed by stronger interactions involving hydrophobic residues in the upper and lower 50-kDa domains (Rayment et al., 1993b; Holmes, 1995). Mutation of regions involved in actin binding might therefore be expected to affect regulation of ATPase activity. Here we show that smooth muscle HMM can be turned into an unregulated, constitutively active molecule simply by replacing its native actin-binding loop (ABL) at the 50/20-kDa junction with that from an unregulated striated muscle myosin.

MATERIALS AND METHODS

Construction of Chimeric Actin-binding Loop HC cDNAs—The polymerase chain reaction (PCR) was used to construct chimeric smooth muscle myosin heavy chain (HC) DNA molecules containing the 50/20-kDa actin-binding loop from chicken skeletal muscle (amino acid sequence GGEAEQGGKGGKGGK) (Maiz et al., 1987) or rat β-cardiac myosin (ANYAGADVDPVKGKKAKK) (McNally et al., 1989). The sequence (residues 626–653) of the native smooth muscle myosin loop is KDVRDGLQDMKAKTEMLSLEKSK (Yanagisawa et al., 1987). For each construct, two overlapping PCR products were deaved internally at a unique common restriction site and ligated. The resulting ~350-base pair chimeric PCR products were cleaved at unique BstEII (nucleotides 1,767–1,773) and AffII (nucleotides 2,019–2,024) sites and ligated as a cassette into an equivalently cleaved chicken gizzard HC cDNA. DNA sequencing verified the fidelity of the inserted PCR sequence.

Infection of Sf9 Cells and Biochemical Methods—Recombinant baculovirus was isolated by conventional protocols (O’Reilly et al., 1992). Sf9 cells in suspension culture were conected with viral particles containing the HMM HC construct (intermediate HMM, 1175-amino acid HC) and a virus expressing both the smooth muscle regulatory (RLC) (Trybus and Chatman, 1993) and essential light chains (Nabeshima et al., 1987). Purification and phosphorylation of the expressed HMM were as described (Trybus, 1994). Immunoblot of glycerol gels were used to verify RLC phosphorylation (Perrie and Perry, 1970).

ATPase and Binding Assays—The concentration of HMM was determined from its NH\text{4}+ ATPase activity relative to a known concentration of purified myosin prepared from turkey gizzard. The buffer for this assay was 0.4 M NH\text{4}Cl, 25 mM Trizma hydrochloride (pH 8.0 at room temperature), 0.2 mM sucrose, 2 mM EDTA, 1 mM dithiothreitol, 4 mM NH\text{4}H\text{2}ATP. Typically, 50–80% of the total protein was active HC. Immunoblot confirmed that the additional bands in the preparation

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‡ The abbreviations used are: HMM, heavy meromyosin; ABL, actin-binding loop at the 50/20-kDa interface of the myosin heavy chain; CABLE, β-cardiac actin-binding loop; SABL, skeletal actin-binding loop; HC, myosin heavy chain; PCR, polymerase chain reaction; RLC, regulatory light chain; WT, wild-type.
are not derived from the myosin HC. To establish that the WT and mutants have the same NH$_4$-ATPase activity, aliquots of protein with equivalent amounts of NH$_4$-ATPase activity were loaded on SDS gels and scanned by densitometry. Within experimental error, bands of equal intensity had equal NH$_4$-ATPase activity. The actin-activated ATPase activity of the expressed constructs is thus based on this concentration determination.

Actin-activated Mg$^{2+}$-ATPase activity was measured as a function of actin concentration in 10 mM imidazole hydrochloride, pH 7.0, 8 mM KCl, 1 mM MgCl$_2$, 1 mM EGTA, 1 mM dithiothreitol, 2 mM MgATP (37°C, 20 μg/ml HMM). Inorganic phosphate was determined colorimetrically (White, 1982).

Binding of mutant and WT-HMMs to actin in the presence of MgATP was determined by a sedimentation assay. A longer tail-length WT-HMM construct of 1329 amino acids was used (Trybus, 1994) to allow the mutant and WT-HMMs to be distinguished in a single gel lane. SABL-HMM and long WT-HMM (0.6 μM heads) were mixed with varying concentrations of actin in the buffer used for ATPase measurements. One μM MgATP was added, and the sample was spun at 350,000 × g for 20 min. Equal volumes of the supernatant and pelleted fractions were run on 9% SDS gels, and the percent of HMM bound was determined densitometrically. Control experiments showed that in the absence of actin essentially all the protein was in the supernatant and that in the absence of MgATP all the protein bound to actin.

Single Turnover Assay—[32P]ATP (0.9 μM; specific activity, 1.8 × 10$^6$ cpm/nmol) was added to HMM (0.46 μM myosin heads) in a buffer containing 25 mM imidazole HCl, pH 7.5, 25 mM KCl, 0.1 mM MgCl$_2$, 1 mM EGTA, and 1 mM dithiothreitol. A 20-fold molar excess of unlabeled ATP was added 10 s later. Nucleotide bound to HMM as a function of time was determined by rapid centrifugation through a dried gel filtration column (Trybus, 1989). NH$_2$-ATPase activity was used to determine the percent recovery of HMM. Control assays were carried out in a buffer containing 0.6 μM KCl, conditions where nucleotide trapping cannot occur.

In Vitro Mobility Assay—Actin filament motility was measured at 30°C as described in Trybus and Chatman (1993). An anti-rod monoclonal antibody (Ab S2; Trybus and Henry, 1989) was used to bind the HMM to the nitrocellulose surface (Trybus, 1994). Where noted, some assays were done at 80 μM KCl, 0.7% methyl cellulose.

**RESULTS**

Dephosphorylated Chimeric Mutants Show High Actin-activated ATPase Activities—Two chimeric smooth muscle HMM mutants, in which the native 50/20-kDa actin-binding loop was substituted with that from skeletal muscle myosin or β-cardiac myosin, were co-expressed with light chains in the baculovirus system. The mutants were named SABL-HMM (Skeletal Actin Binding Loop) and CABL-HMM (Cardiac Actin Binding Loop) (see "Materials and Methods" for sequences). SABL-HMM and CABL-HMM are indistinguishable from WT-HMM in their heavy and light chain complement (Fig. 1A).

The actin-activated ATPase activity of dephosphorylated and phosphorylated WT-HMM, SABL-HMM, and CABL-HMM were determined (Fig. 2, A–C). The extrapolated $V_{\text{max}}$ values obtained from the illustrated best fit curves for the phosphorylated species are: 2.6 ± 0.12 s$^{-1}$ for SABL-HMM, 3.7 ± 0.68 s$^{-1}$ for phosphorylated CABL-HMM, and 4.2 ± 1.1 s$^{-1}$ for phosphorylated WT-HMM. These ATPase activities do not reflect the higher activity of the myosin from which the loop sequence was derived.

Surprisingly, the major effect of the loop mutations was to activate the dephosphorylated species. The $V_{\text{max}}$ of dephosphorylated SABL-HMM (3.1 ± 0.34 s$^{-1}$) was virtually identical to its phosphorylated counterpart, while dephosphorylated CABL-HMM had a $V_{\text{max}}$ of 2.0 ± 0.45 s$^{-1}$, only 2-fold less than phosphorylated CABL-HMM. Immunoblots of the samples used for the ATPase assay showed that the levels of phosphorylation in the phosphatase-treated samples were negligible (Fig. 1C).

The dephosphorylated chimeric mutants had a 3-4-fold lower $K_{m}$ for actin than phosphorylated WT-HMM (see Fig. 2 legend for values). Phosphorylation decreased the $K_{m}$ further to <1 μM for phosphorylated SABL-HMM. Thus at low actin concentrations, the apparent effect of the ABL mutations is to increase activity, but this trend does not persist when the values are extrapolated to $V_{\text{max}}$.

WT and SABL-HMM Bind to Actin with Equal Affinity—To determine if the activation of the dephosphorylated HMMs is due to an increase in binding of the chimeras to actin, a sedimentation assay was performed (Fig. 1B and Fig. 2D). The results show that the binding of dephosphorylated WT-HMM and dephosphorylated SABL-HMM to actin in the presence of MgATP was indistinguishable. The binding constant was ap-
proximately \(8 \times 10^3 \text{M}^{-1}\), consistent with earlier measurements with chymotryptic HMM (Sellers et al., 1982). Activation must therefore result from changes to a kinetic step other than binding to actin.

SABL-HMM Traps Nucleotide in the Absence of Actin—To determine whether the ABL chimeras were also unregulated in the absence of actin, single-turnover assays were performed on dephosphorylated WT-HMM and SABL-HMM (Fig. 3). SABL-HMM was chosen because its actin-activated ATPase activity was completely unregulated. WT-HMM trapped nucleotide in low ionic strength buffer (25 mM KCl), releasing hydrolyzed Pi was completely unregulated. WT-HMM trapped nucleotide in the absence of actin, single-turnover assays were performed on dephosphorylated WT-HMM and SABL-HMM (Fig. 3). SABL-HMM released nucleotide at an ionic strength more closely approximating physiological conditions.

Actin Movement by Chimeric Mutants Is Not Regulated by RLC Phosphorylation—Dephosphorylated SABL and CABL-HMMs both gained the ability to move actin in an in vitro motility assay (Fig. 4). The velocity of movement by SABL-HMM was independent of phosphorylation, while the velocity of phosphorylated CABL-HMM was only 1.5-fold higher than dephosphorylated CABL-HMM. Thus the degree of regulation found in the motility assays was similar to that observed in the ATPase measurements. Dephosphorylated WT-HMM showed no movement under the conditions of our assay and thus was completely regulated. One motility experiment conducted at 60 mM KCl showed that dephosphorylated SABL-HMM was still active at an ionic strength more closely approximating physiological conditions.

The chimeric substitutions caused a decrease in the velocity of actin filament movement by the phosphorylated HMM species, from 1.1 \(\mu\text{m/s}\) with WT-HMM to 0.34 \(\mu\text{m/s}\) with SABL-HMM and 0.80 \(\mu\text{m/s}\) with CABL-HMM. The motility with SABL-HMM was about 2-fold lower than would be expected based on the ATPase measurements, which were 60–70% of phosphorylated WT-HMM.

DISCUSSION

The sequence of the ABL at the 50/20-kDa junction in the myosin head is highly divergent, suggesting that it could confer isoform-specific properties onto myosin. Here we show that the native sequence of the smooth muscle myosin ABL is required for regulation by light chain phosphorylation. Replacement of the native ABL with that from unregulated fast skeletal (SABL-HMM) or \(\beta\)-cardiac myosin (CABL-HMM) caused dephosphorylation of HMM, which is normally in the “off” state, to proceed through its ATPase cycle with at least 50% the rate of phosphorylated WT-HMM and move actin in a motility assay.

How can a loop at the opposite end of the head from the RLC affect regulation? Loss of regulation implies that inorganic phosphate can no longer be trapped at the active site in the presence of actin. The ABL straddles the cleft that separates the upper and lower 50-kDa domains (Raymond et al., 1993a) and may be instrumental in initiating a multistep process that leads to cleft closure and release of phosphate through the “back door” (Yount et al., 1995). Thus there is a fairly direct line of communication between the ABL and the phosphate-binding site at the base of the cleft in the 50-kDa domain, and it is reasonable that changing the way actin interacts with a given ABL could inhibit or facilitate cleft closure and hence affect regulation. Continuity between the ABL, the nucleotide-binding site, and the RLC is maintained through the 20-kDa portion of the HC, which traverses the length of the head; the ABL is adjacent to the N terminus of the 20-kDa segment, which continues past the nucleotide site, and ends in a long \(\alpha\)-helix to which the LCs bind (Raymond et al., 1993a). The balance between the two lines of communication, RLC ↔ active site ↔ ABL, therefore determines whether or not phosphate is released.

Spadich and colleagues (Uyeda et al., 1994) suggested that the 50/20-kDa loop affects the rate-limiting step of myosin’s ATPase cycle, based on the observation that a chimeric phosphorylated Dictyostelium myosin with a fast skeletal ABL had a 5-fold higher actin-activated ATPase than WT myosin. Because the actin-activated ATPase activity of phosphorylated smooth muscle SABL or CABL-HMM was not enhanced, this role for the loop is not a general feature of all myosin motors.

The ABL mutations decreased the \(K_m\) for actin, resulting in an apparent activation at low actin concentrations, but this increase does not persist at high actin concentrations.

Activation of the dephosphorylated chimeras was not due to an increased affinity for actin in the presence of MgATP. This result implies that the foreign loops increase the rate of a transition other than binding. The molecular step is probably the same as that activated by RLC phosphorylation in the native molecule, which is phosphate release or a step just preceding release but following hydrolysis (Sellers et al., 1982). The similarity in the \(V_{\text{max}}\) values for the dephosphorylated and phosphorylated chimeras argues for this interpretation.

Introduction of the ABL loop into smooth muscle HMM must also affect the rate of other kinetic transitions in the ATPase cycle. The \(K_m\) for SABL-HMM is significantly lower than for phosphorylated WT-HMM. The leftward shift in \(K_m\) upon phosphorylation of SABL-HMM is consistent with the observation that phosphorylation increases binding to actin (Sellers et al., 1982). Since more than one elementary rate constant contributes to \(K_m\) in a model-dependent way (Taylor, 1979), we cannot speculate at present as to which step is altered by the intro-

**Fig. 3.** Release of \(^{32}\text{P}\) in a single turnover from dephosphorylated SABL-HMM. Data were obtained at 25 mM KCl (filled circles) and 0.6 M KCl (open circles). Rate constants were obtained using the best fit equation of the form, \(y = N_0 e^{-kt}\), where \(N_0\) is moles of phosphate bound per mol of HMM head at \(t = 0\), \(k\) is the rate constant, and \(t\) is time.

**Fig. 4.** Velocity of actin movement by phosphorylated and dephosphorylated HMM. All values are mean velocities ± S.D. for three independent preparations of SABL-HMM, two of CABL-HMM, and four of WT-HMM. For each preparation, 12 filaments were measured to obtain an average velocity for each condition. Dephosphorylated (de\(P\)) WT-HMM did not show any measurable motility.
duction of chimeric ABLs. The reduced rate of motility with SABL-HMM implies that ADP release (Siemankowski et al., 1985) may also be affected by the skeletal loop sequence.

Regulation of activity in smooth muscle myosin also occurs in the absence of actin. The most striking example of this is the "trapping" of nucleotide at the active site when myosin adopts the folded monomeric conformation (Cross et al., 1986), but chymotryptic HMM (Sellers, 1985) and dephosphorylated myosin filaments (Trybus, 1989) also show a strong inhibition of product release. Dephosphorylated SABL-HMM retained the ability to trap products, showing that foreign loops facilitate phosphate release only in the presence of actin. The ABL is likely to be disordered and flexible since it is not seen in the crystal structure (Rayment et al., 1993a) and can be readily proteolyzed (Mornet et al., 1981). When it interacts with actin, the ABL probably adopts a more ordered structure and thereby increases its influence on the active site.

Nineteen of the 26 residues in the actin-binding loops from myosin regulated by light chain phosphorylation are identical. These differences did not cause an increase in binding affinity to actin, and 2) the smooth ABL is longer (26 residues) than that from either cardiac or skeletal muscle myosin (both +3), although this charge difference did not cause an increase in binding affinity to actin. Nineteen of the 26 residues in the actin-binding loops from smooth muscle myosin ABL (+2) is less than that from the actin-binding loop. The sequence of the actin-binding loop from smooth muscle myosin has been conserved so that its binding to actin does not overcome the inhibition of product release imposed by a dephosphorylated RLC. Regulation is thus an intricate mechanism of how two spatially distant sites, the RLC and the actinomyosin interface, communicate with the active site.

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