Sites of Interaction between Kinase-related Protein and Smooth Muscle Myosin*

Debra L. Silver‡, Alexander V. Vorotnikov‡, D. Martin Wattersong¶, Vladimir P. Shirinsky§, and James R. Sellers**

From the ‡Laboratory of Molecular Cardiology, NHLBI, Bethesda, Maryland 20892, the §Laboratory of Molecular Endocrinology, Institute of Experimental Cardiology, Russian Cardiology Research Centre, Moscow 121552, Russia, and the ¶Drug Discovery Program and Department of Molecular Pharmacology and Biological Chemistry, Northwestern University, Chicago, Illinois 60611

Kinase-related protein, also known as KRP or telokin, is an independently expressed protein product derived from a gene within the gene for myosin light chain kinase (MLCK). KRP binds to unphosphorylated smooth muscle myosin filaments and stabilizes them against ATP-induced depolymerization in vitro. KRP competes with MLCK for binding to myosin, suggesting that both proteins bind to myosin by the KRP domain (Shirinsky, V. P., Vorotnikov, A. V., Birukov, K. G., Nanaev, A. K., Collinge, M., Lukas, T. J., Sellers, J. R., and Watterson, D. M. (1993) J. Biol. Chem. 268, 16578–16583). In this study, we investigated which regions of myosin and KRP interact in vitro. Using cosedimentation assays, we determined that KRP binds to unphosphorylated myosin with a stoichiometry of 1 mol of KRP/1 mol of myosin and an affinity of 5.5 μM. KRP slows the rate of proteolytic cleavage of the head-tail junction of heavy meromyosin by papain and chymotrypsin, suggesting it is binding to this region of myosin. In addition, competition experiments, using soluble headless fragments of nonmuscle myosin, confirmed that KRP interacts with the regulatory light chain binding region of myosin. The regions important for KRP’s binding to myosin were investigated using bacterially expressed KRP truncation mutants. We determined that the acid-rich sequence between Gly138 and Asp151 of KRP is required for high affinity myosin binding, and that the amino terminus and β-barrel regions weakly interact with myosin. All KRP truncations, at concentrations comparable to their KD values, exhibited some stabilization of myosin filaments against ATP depolymerization in vitro, suggesting that KRP’s ability to stabilize myosin filaments is commensurate with its myosin binding affinity. KRP weakened the K0, but not the Vmax of phosphorylation of myosin by MLCK, demonstrating that bound KRP does not prevent MLCK from activating myosin.

In resting smooth muscle, myosin exists in a predominantly dephosphorylated and inactive state (1) and contraction is initiated by a calcium-calmodulin-dependent phosphorylation of the regulatory light chain (RLC)1 of myosin by myosin light chain kinase (MLCK) (2). In vitro, phosphorylation of myosin activates its MgATPase activity in the presence of actin and also allows myosin to translocate actin filaments (3). Phosphorylation also affects the equilibrium between filamentous and monomeric myosins in vitro (4). Filaments formed from unphosphorylated myosin are dissociated by MgATP into a monomeric form of myosin in which the tail forms a hairpin fold back onto the neck region of the myosin head. This folded monomeric myosin has been termed “10 S” myosin based on its sedimentation coefficient in the analytical ultracentrifuge, while phosphorylated monomeric myosin exists primarily in an extended conformation, called “6 S” (5, 6). Phosphorylation of the RLC greatly stabilizes filaments against MgATP dissociation (4, 7).

In vivo, smooth muscle myosin filaments appear to be stable even under conditions where myosin is predominantly unphosphorylated (1, 8, 9). The discrepancy between myosin’s filamentous state in vivo and in vitro may be explained by the presence of regulatory proteins, which stabilize unphosphorylated smooth muscle myosin filaments in vivo. One candidate for such a protein is an abundant myosin-binding protein, termed kinase-related protein (KRP) and also referred to as telokin. KRP binds to unphosphorylated myosin filaments in vitro and stabilizes them against MgATP-dependent depolymerization (10). KRP binds tightly to the heavy meromyosin (HMM), but not to the subfragment-1 (S1) or rod domains of myosin, suggesting that KRP’s binding site is in the S1-S2 region of myosin. In addition, KRP may prevent myosin from adopting the 10 S conformation, by competing for the region on myosin where the tail folds over, which is thought to include the RLC binding site (11).

The gene encoding KRP is nested within the gene for MLCK, and, in fact, the 157-residue sequence of KRP is completely identical to the sequence of the carboxyl terminus of MLCK (12, 13). Western analyses estimate that in avian gizzard smooth muscle, KRP is abundant compared with MLCK, and is approximately stoichiometric with myosin (10, 14). It is likely that MLCK interacts with myosin primarily by its KRP domain, since KRP and MLCK compete for myosin binding and a truncated

* This work was supported in part by Howard Hughes Medical Institute Grant 75195-546901 (to V. P. S.), RFFI Grant 96-04-49106 (to A. V. V.), and National Institutes of Health Grant GM30861 (to D. M. W.). 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 1734 solely to indicate this fact.

‡John G. Searle Professor of Molecular Biology and Biochemistry.

**To whom all correspondence should be addressed: Laboratory of Molecular Cardiology, NHLBI, Bldg. 10, Rm. 8N202, 10 Center Dr., MSC 1762, Bethesda, MD 20892-1762. Tel: 301-496-6887; Fax: 301-402-1542; E-mail: jsellers@helix.nih.gov.

1 The abbreviations used are: RLC, regulatory light chain; MLCK, myosin light chain kinase; ELC, essential light chain; HMM, heavy meromyosin; LMM, light meromyosin; S1, subfragment-1; S2, subfragment-2; FKA, cyclic AMP-dependent kinase; MAPK, mitogen-activated kinase; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; MOPS, 3-(N-morpholino)propanesulfonic acid; BSA, bovine serum albumin protein; KRP, kinase-related protein; PCD, polymerase chain reaction.
cated MLCK that lacks the KRP domain cannot bind myosin as well as intact MLCK (10).

The crystal structure of KRP has been solved and shows that the protein is a seven-stranded C2 immunoglobulin-like β-barrel with trilayered walls at either end (15). The amino-terminal 35 amino acids and the carboxyl-terminal 19 amino acids were not resolved in the crystal structure, suggesting that they are in an unstructured or flexible conformation. The amino terminus contains two putative sites for phosphorylation by cyclic AMP-dependent kinase (PKA) and mitogen-activated protein kinase (MAPK) (16), while the carboxyl terminus is distinguished by a high content of acidic residues. To determine the sites of interaction between KRP and myosin, we have quantified KRP binding to myosin and examined its interaction with various proteolytic and recombinant fragments of myosin. We show that KRP binds at the RLC region of the myosin heavy chain, probably where the two heads join to form the rod region of myosin. We have expressed amino- and carboxyl-terminal truncation mutants of KRP and studied their binding to myosin. Furthermore, we demonstrate that the acidic carboxyl terminus of KRP is important for high affinity myosin binding and subsequent stabilization of myosin filaments. Finally, we show that KRP affects the K_m, but not the V_max for phosphorylation of myosin by MLCK. Some of these data have been presented in preliminary form (17).

EXPERIMENTAL PROCEDURES

Protein Preparation—KRP (18), smooth muscle myosin (19), HMM (20), MLCK (14), and S1 (21) were purified as described from fresh or frozen turkey or chicken gizzards. PKA was prepared according to the method of Sugden et al. (22). Soluble headless fragments of nonmuscle myosin IIB were expressed using the baculovirus system and purified using a carboxyl terminus FLAG-tag. Fragments corresponded to subfragment 2 (S2) plus 1, or 2 light chain binding sites and associated light chains.2

Bacterial Expression and Purification—KRP was expressed in bacteria using a nonmuscle MLCK cDNA clone corresponding to nucleotides 3966–4436 of cDNA sequence. KRP truncations were created by restriction enzymes (New England Biolabs, Beverly, MA) and gel-purified (Qiagen extraction kit, Qiagen, Chatsworth, CA). A Nco I and Xho I restriction enzyme combination was used for the expression of KRP cDNA. The truncations were ligated into the vector pET-28a and electrophoresed on 12.5% SDS-polyacrylamide gels. Coomassie blue-stained bands were excised from gels and proteins representing the desired fragments were excised from the gel. KRP truncations were further purified by metal affinity chromatography. KRP truncations were eluted using 2 volumes of elution buffer (M HMM was incubated with 22 μM KRP in buffer A (without ATP) at 25 °C for 5 min. The reaction was initiated with the addition of 0.3 mg/ml PKA with 15 μg/ml PKA, 0.5 mM γ-32P[ATP], in 50 mM NaCl, 10 mM Mops (pH 7.0), 25 mM MgCl2, 0.1 mM EDTA, 3 mM Na3VO4, and 3 mM DTT.

Protein Concentration Determination—Protein concentrations were measured spectrophotometrically (Beckman, Columbia, MD). For KRP, the following extinction coefficients were calculated according to Gill and von Hippel (23): 0.74, 0.73, 0.77, 0.85, 0.73, 0.78, 0.98, 1.08, and 0.69, for tissue-purified KRP and truncations 1–5, respectively.

Cosedimentation Assays—Binding affinity and stoichiometry of wild type KRP were determined by using PKA-phosphorylated [32P]KRP (tissue-purified). [32P]KRP was prepared by incubation of 0.3 mg/ml KRP with 15 μg/ml PKA, 0.5 mM γ-32P[ATP], in 50 mM NaCl, 10 mM MOPS (pH 7.0), 25 mM MgCl2, 0.1 mM EDTA, 3 mM Na3VO4, and 10 mM DTT, for 1 h at 25 °C, followed by exhaustive dialysis against buffer A. Varying concentrations of myosin and [32P]KRP were incubated in 50 μl of buffer A for 15 min at 25 °C. The reaction mixture was spotted at 436,000 × g for 10 min at 25 °C in a TLA 100 rotor in the Optima TL ultracentrifuge (Beckman, Palo Alto, CA). Pellets were counted in a scintillation counter (Beckman LS6001, Columbia, MD) and mol of [32P]KRP bound/mol of myosin was determined. The binding affinity and stoichiometry were calculated by fitting the data to the equation: b = (B_max S/K_m + S), where S = concentration of [32P]KRP, B_max = mol of [32P]KRP/mol of myosin, and K_m = dissociation constant.

For bacterially expressed KRP truncations, nonradioactive samples were incubated and cosedimented as above, with myosin and 0.1 mg/ml bovine serum albumin (BSA), which was used as a volume marker for the pellet. Binding was analyzed by gel electrophoresis on 12.5% SDS-polyacrylamide gels. The supernatants (40 μl) were collected every 30 s for 5 min, and the reaction was stopped with 200 units of proteinase K (24). For papain digestions, 5 μM KRP was incubated with 22 μM KRP in buffer A (without ATP) at 25 °C for 5 min. The reaction was initiated with the addition of 200 units of papain/mg of myosin ( Worthington, Freehold, NJ) in papain activating solution (0.1 mM NaCl, 10 mM NaPO4, 0.2 mM EDTA, pH 7.0) Time points were collected every 30 s for 5 min, and the reaction was stopped with 20 mM iodoacetate. Samples were added to 2 × sample buffer, boiled, and electrophoresed on 12.5% SDS-polyacrylamide gels. Coomassie Blue-stained gels were quantitated by densitometry, and the digestion was monitored by the relative amount of S2 produced at each time point. Data were generated from four experiments.

For chymotrypsin digestions, 3.7 μM KRP was incubated with 22 μM KRP in buffer A (without ATP) at 25 °C for 5 min. The reaction was initiated with the addition of 0.1 mg/ml papain (Sigma) to a 1:20 ratio (w/w) of protease to KRP, and time points were collected at 5, 10, 20, 40, and 80 min. The digestion was terminated with 1 mM PMSF. Samples were electrophoresed and quantitated by densitometry (NIH Image, Bethesda, MD) of the amount of RLC digested, with normalization to undigested essential light chain (ELC). Data were generated from three experiments.

ATPase Assays—The protection of myosin filaments against ATP-depolymerization by KRP truncations was monitored by measurement of the (NH4)2EDTA-ATP activities of soluble myosin. Myosin purified from chicken gizzards was used for these experiments, as this source of myosin was consistently soluble upon addition of 2 mM MgATP.
Myosin filaments were prepared by dialysis against 150 mM NaCl, 10 mM MOPS (pH 7.0), 1 mM MgCl₂, 3 mM NaN₃, and 3 mM DTT. 3 μM myosin filaments and 15 μM KRP truncations were incubated in buffer A brought to 100 mM NaCl in the presence of 2 mM MgATP, and cosedimented as above. Following centrifugation, 5 μL of the supernatants was added to the following ATPase mixture: 0.4 mM NH₄Cl, 35 mM EDTA, 30 mM NaCl, 25 mM HEPES (pH 8.0), 2 mM MOPS, 0.2 mM MgCl₂, 0.2 mM EGTA, 5 mM [γ-³²P]ATP, 0.5 mg/ml BSA. The rate of phosphate release was measured at 25 °C over 15 min according to Sellers et al. (20). Samples were measured in duplicate, and the percent of soluble myosin was determined by normalization to the myosin control without KRP.

HMM Phosphorylation by MLCK—HMM, ranging in concentration from 1 to 18 μM, was phosphorylated in buffer A with 0.5 mM [γ-³²P]ATP and 1 nM MLCK, according to the procedure of Sellers et al. (20), in the presence or absence of 50 μM KRP. The values for Kₘ and Vₘₐₓ were determined from fits to the Michaelis-Menten equation (Enzfitter, London, United Kingdom).

**RESULTS**

**KRP Binding Affinity and Stoichiometry**—Studies of KRP binding to myosin were performed by a cosedimentation assay, using conditions in which KRP is soluble and myosin is filamentous and sedimentable. KRP has two sites for phosphorylation by PKA and MAPK, respectively. PKA-phosphorylated KRP has the same binding capacity as unphosphorylated KRP (16). Therefore, [³²P]KRP was used in binding studies to determine KRP’s binding affinity and stoichiometry of binding to myosin. The Kₐ was 5.5 μM, and a stoichiometry of 1.10 mol of KRP/mol of myosin molecule was determined (Fig. 1).

**KRP Binds to the S1-S2 Domain of Myosin**—Previous experiments showed that KRP binds well to unphosphorylated HMM, but not to the S1 or rod domains of myosin (10). In addition, the ability of KRP to protect myosin against ATP-induced depolymerization in vitro suggested that KRP may prevent myosin from adopting the 10 S (folded) conformation, which is thought to occur by binding of the rod of myosin to its S1-S2 junction (6, 11). We examined whether KRP binds to this site by using proteolytic protection assays. Papain will proteolytically digest HMM into S1 and S2 fragments (25). Fig. 2A depicts a time course of this reaction in the presence and absence of KRP. Inclusion of KRP retarded the rate of papain digestion of the heavy chain of HMM and also inhibited the rate of proteolysis of the RLC. In Fig. 2B, this effect is quantified as the proportion of S2 produced over time. KRP also afforded protection of the RLC of HMM against chymotrypsin digestion (Fig. 2C). In both experiments, KRP itself was not digested, indicating that it was not merely acting as a competitor for proteolysis by the enzymes.

**Competition Experiments Reveal Binding to the RLC Binding Region**—KRP protection of myosin against proteolysis suggested that it bound to the S1-S2 junction of myosin. Since KRP

---

**Graphs and Figures**

**Fig. 1. Binding of KRP to myosin.** [³²P]KRP (tissue-purified) was cosedimented with smooth muscle myosin under low salt buffer conditions (buffer A), and mol/mol bound was determined for each [³²P]KRP concentration. The data were fit to the following equation: $b = (B_{\text{max}} + S/K_{D})/S$, where $B_{\text{max}}$ = mol/mol bound, $S$ = [³²P]KRP concentration, and $K_{D}$ = dissociation constant. A $K_{D}$ of 5.5 μM and a $B_{\text{max}}$ of 1.10 were determined.

**Fig. 2. KRP protects the myosin heavy chain and the RLC against proteolysis at the S1-S2 junction.** HMM was digested by papain and chymotrypsin with and without KRP present. A, two 12.5% SDS-polyacrylamide gels, which represent samples from 11-min papain digestions. B, quantification of the rate of papain digestion of the myosin heavy chain, depicted by the amounts of S2 produced over time. C, quantification of the rate of chymotryptic digestion of the RLC.
bound similarly to nonmuscle and smooth muscle myosins (data not shown), we used nonmuscle myosin fragments containing this region in competition binding experiments, to more precisely localize KRP’s binding site. Soluble headless fragments of nonmuscle myosin IIB corresponding to S2, S2 and bound RLC, and S2 and bound RLC and ELC were prepared by baculovirus expression (Fig. 3A). The soluble fragments were included in cosedimentation assays with smooth muscle myosin and KRP, and the depletion of KRP from the pellet indicated competitive binding to the soluble fragment. The two constructs containing the RLC binding site competed strongly with myosin for KRP binding, while S2 alone did not compete as well, indicating a weaker interaction with KRP (Fig. 3B).

Expression of KRP Truncations in Bacteria—Truncation mutants of KRP were made by PCR of a nonmuscle MLCK cDNA template. The truncations were designed based upon the crystal structure previously determined (15) and the primary structure, as shown in Fig. 4 (A and B). The three-dimensional structure of KRP consists of a core seven-stranded β-barrel with a C2-type IgG motif. The amino- and carboxyl-terminal tails, containing amino acids 1–35 and 138–157, respectively, were not resolved in the crystal structure, presumably due to their flexibility. The consensus sites for phosphorylation by PKA and MAPK are found within the amino terminus, and an acid-rich domain characterizes the carboxyl terminus (13 of 17 residues are acidic). Truncations were created to understand the significance of these regions for myosin binding and stabilization properties in vitro. Fig. 5A is a schematic representation of the expressed truncations, and their migration on a 20% SDS-polyacrylamide gel is shown in Fig. 5B. Since preparations of truncations 5, 6, and 7 consistently migrated as more than one band, the integrity of these truncations was confirmed by mass spectroscopy. This method revealed the expected sequences with only a methionine missing in some cases, suggesting that the extra bands may be due to intramolecular bonds (data not shown).

The COOH Terminus of KRP Is Significant for Myosin Binding and Filament Stabilization in Vitro—The KRP truncations were assayed for their myosin binding capacities using cosedimentation assays. The binding affinities ($K_D$, dissociation constant) of the KRP truncations are indicated in Table I. Truncation of the first 23 residues at the amino terminus, K(12–157) and K(23–157), did not affect myosin binding, while truncation of the first 36 residues, K(36–157), weakened the binding slightly. Truncation of 7 residues at the carboxyl terminus, K(1–150), also did not affect binding affinity, whereas removal of 19 residues at the carboxyl terminus, K(1–138), weakened KRP binding by about 5-fold. The smallest truncations, representing the amino acids resolved in the crystal structure, K(36–138), and the core β-barrel, K(43–136), also had weaker binding affinities.

KRP truncations (15 μM) exhibited a similar pattern in their abilities to protect against the depolymerization of myosin (3 μM) by MgATP in vitro. All three amino-terminal KRP truncations, K(12–157), K(23–157), and K(36–157), stabilized myosin filaments like wild type KRP. However, all of the carboxyl-terminal truncations, K(1–138), K(36–138), and K(43–136) which lack the acidic region, failed to stabilize myosin filaments (Table I). Increasing the concentration of these weaker

---

**Fig. 3.** Soluble myosin fragments containing RLC compete with myosin for KRP binding. Smooth muscle myosin and KRP (1:3 molar ratio) were cosedimented with molar excesses of soluble nonmuscle myosin fragments, depicted schematically in A. The black box represents the binding site for ELC; the shaded box, the binding site for RLC; and the open box, the S2 region. In B, the percent competition is the proportion of KRP binding to smooth muscle myosin in the presence of fragments versus KRP binding to myosin in their absence. Only nonmuscle myosin fragments containing RLC effectively competed with full-length myosin for KRP binding.

**Fig. 4.** Crystal structure and primary sequence of KRP. A, the KRP core is a seven-sheet β-barrel of a C2 immunoglobulin-like motif (15). The structure is depicted here with the amino terminus at the right and the carboxyl terminus at the left. The amino-terminal 35 residues and the carboxyl-terminal 19 residues were unresolved in the crystal structure. B, the KRP primary sequence is 157 amino acids. Those residues resolved in the crystal structure are indicated here in bold, and the phosphorylation sites for PKA and MAPK are underlined.

---

**Table I.** The COOH Terminus of KRP Is Significant for Myosin Binding and Filament Stabilization in Vitro

| Construct   | Amino Acids | Myosin Binding | Filament Stabilization |
|-------------|-------------|----------------|------------------------|
| S2          | 1–150       | Low            | Low                    |
| S2+RC       | 1–138       | Moderate       | Moderate               |
| S2+RC+ELC   | 1–138       | High           | High                   |

---

**Table II.** Interaction Sites between KRP and Myosin

| Interaction Site | Amino Acids |
|-----------------|-------------|
| PKA             | 12–157      |
| MAP             | 23–157      |
| RLC             | 136–157     |

---

**Fig. 5.** SDS-PAGE analysis of KRP truncations. A, a 20% SDS-polyacrylamide gel showing the migration of the expressed truncations, and their migration on a 20% SDS-polyacrylamide gel is shown in Fig. 5B. Since preparations of truncations 5, 6, and 7 consistently migrated as more than one band, the integrity of these truncations was confirmed by mass spectroscopy. This method revealed the expected sequences with only a methionine missing in some cases, suggesting that the extra bands may be due to intramolecular bonds (data not shown).
binding truncation mutants to levels where binding was comparable to $K_D$ led to further stabilization. For example, inclusion of 60 mM of K(1–138) and K(36–138) left 45% and 18% myosin soluble, respectively. The relative abilities of KRP truncations to stabilize myosin filaments in vitro appears to be proportional to their binding affinities. Altogether the data confirm that KRP binds myosin with high affinity by its acidic tail and weakly interacts with myosin by its amino terminus and core $\beta$-barrel.

**Effect of KRP on Phosphorylation of HMM by MLCK**—The evidence presented above suggests that KRP binds to the S1-S2 junction, probably in the vicinity of the phosphorylatable residue on the RLC and that this region is likely the same site to which MLCK binds. This raises the possibility that KRP might act as a competitive inhibitor of RLC phosphorylation. To directly examine this prospect, the steady state kinetics of phosphorylation of HMM was measured in the absence and presence of a near saturating concentration of KRP (50 mM). The data show that KRP increases the $K_m$ for phosphorylation approximately 10-fold, but does not dramatically affect the $V_{max}$ (Fig. 6).

**Table I**

| KRP construct | KRP binding ($K_D$) μM | Filament stability % soluble |
|---------------|------------------------|-----------------------------|
| WT            | 9.5                    | 13 ± 1.0                    |
| 1 K(12–157)   | 12.2                   | 5 ± 0.9                     |
| 2 K(23–157)   | 13.7                   | 7 ± 0.9                     |
| 3 K(36–157)   | 27.5                   | 17 ± 4.0                    |
| 4 K(1–150)    | 7.3                    | 18 ± 5.0                    |
| 5 K(1–138)    | 46.0                   | 67 ± 8.0                    |
| 6 K(36–138)   | 42.5                   | 59 ± 3.0                    |
| 7 K(43–136)   | 62.0                   | 80 ± 9.0                    |

**Fig. 5.** Bacterial expression of KRP mutant truncations. A, PCR-generated truncations are depicted schematically. B, a 20% SDS-polyacrylamide gel depicts tissue-purified KRP, mutants 1–8, respectively.

**Fig. 6.** KRP affects the $K_m$ but not the $V_{max}$ of phosphorylation of HMM by MLCK. HMM phosphorylated in the absence of KRP (open circles) has a measured $K_m$ value of 2.4 μM and a $V_{max}$ of 2.1 μmol/min/mg. HMM phosphorylated in the presence of KRP (closed circles) has a $K_m$ value of 29 μM and a $V_{max}$ of 1.8 μmol/min/mg.

**DISCUSSION**

A knowledge of the binding sites between KRP and myosin is necessary for understanding how KRP stabilizes myosin filaments and elucidating the structural and functional relationships between MLCK and KRP. The question of how KRP binds myosin was especially intriguing to address because of the shared structural motifs in KRP and MLCK. The predominant structure of KRP is a seven-stranded C2-type immunoglobulin (IgG)-like motif (15). The amino- and carboxyl termini of KRP, which were not resolved in the crystal structure, are also characterized by interesting sequences, including phosphorylation sites at the amino terminus of KRP (16), and a highly acidic carboxyl terminus (13 of 17 residues).

MLCK has two other IgG repeats, in addition to the KRP IgG repeat, which are located amino-terminal to the catalytic core (26). None of these IgG repeats are essential for MLCK catalysis (27). IgG repeats are found in other myosin-binding pro-
teins. MyBP-C, also called C-protein, is a skeletal muscle thick filament protein, which contains seven copies of the IgG motif (28) and can bind to LMM and S2 (29, 30). The carboxy-terminal IgG motif is responsible for high affinity binding to myosin (31, 32). Moreover, Okagawa et al. (31) compared the primary sequences of the myosin binding IgG motif in MyBP-C and the carboxy-terminal IgG motif of MLCK (the KRP domain), and predicted 15 residues within the KRP β-barrel that might be important for binding to myosin. Another myosin-binding protein is vertebrate twitchin, which is located mostly throughout the I band domain, but uses several IgG motifs within the A band to interact with LMM (35, 36). A titin homologue in C. elegans, twitchin, contains 26 IgG motifs (37). Interestingly, the carboxyl terminus of twitchin is organized into domains that are similar in protein sequence and arrangement to MLCK (38). Recently, Kobe et al. (38) used the crystal structure of the twitchin carboxy-terminal IgG motif to model the corresponding structure in MLCK (the KRP domain). Based on this model and the myosin binding sequences that were predicted by Okagawa et al. (31), the authors specified 6 residues within the KRP β-barrel, Phe44, Asp50, Val82, Val118, Glu123, Ala124, which might participate in binding to myosin. In addition, since titin uses one of its IgG motifs to bind to skeletal LMM (35), Kobe et al. (38) suggested that MLCK might interact with the homologous residues, Leu1824–Arg1840, in the LMM portion of smooth muscle myosin.

This proposed model for KRP binding to myosin is inconsistent with the results obtained here. In our in vitro assays with bacterially expressed KRP truncation mutants indicated that the acid-rich carboxy terminus of KRP, corresponding to the sequence Gly138–Glu150, is the primary determinant of KRP’s binding affinity for myosin. The Kobe et al. (38) model postulates that important residues for myosin binding are contained within the KRP β-barrel, but we found that truncation mutants representing amino acids resolved in the crystal structure, K(36–138), and the core β-barrel, K(43–136), bound only weakly to myosin. However, it is important to note that the carboxyl terminus truncations could at least partially stabilize myosin filaments in the presence of ATP, if sufficiently high concentrations were used to obtain binding. The amino terminus is not critical for interaction with myosin, as indicated by our binding assays with truncations and by the lack of effects of phosphorylation of KRP on its binding to myosin (16). These assays suggest that the KRP β-barrel and the amino-terminal extension do not determine the high affinity binding to myosin, but that the β-barrel does weakly bind to myosin.

In addition, we have shown that KRP clearly does not bind to the region of smooth muscle myosin postulated by Kobe et al. (38). It should be noted that there are numerous examples of IgG motifs that do not bind LMM, such as the two amino-terminal IgG motifs of MLCK, which do not appear to bind myosin (27).

Evidence from Sellers and Pato (19), Shirinsky et al. (10), and the present study indicates that KRP and MLCK share a primary myosin docking site at the S1-S2 junction determined by the KRP domain that is independent of MLCK’s catalytic interaction with the RLC. The idea that MLCK possesses both a catalytic binding site and a “docking” site that is shared with KRP is supported by the steady state kinetic analysis of HMM phosphorylation, which clearly demonstrates that KRP binding to myosin only affects the Km and not the Vmax of phosphorylation of myosin by MLCK. Since the KRP binding site is not required for catalysis, MLCK could phosphorylate myosin light chains even if KRP is bound, but MLCK now can only bind via its catalytic site. However, because KRP is more abundant in a smooth muscle cell, and evidence suggests that MLCK predominantly associates with actin in vivo (39, 40), it is likely that more myosin molecules have KRP bound than MLCK. Nevertheless, MLCK should be able to efficiently phosphorylate myosin.

KRP binding to the S1-S2 junction of myosin is consistent with its apparent ability to shift the myosin equilibrium from the 10 S to the 6 S conformation in vitro. This region has been implicated in the formation of myosin in the folded 10 S conformation. A peptide corresponding to 12 residues, Leu835–Lys840, from the head/neck junction of the myosin heavy chain, inhibits the formation of the 10 S conformation (41). This is the location where we propose KRP binds. KRP’s ability to protect myosin against papain proteolysis at the S1-S2 junction is also strikingly similar to the resistance of both the heavy chains and light chains to digestion when myosin is in the 10 S conformation (42, 43). In addition, during the preparation of this manuscript, a paper was published reporting the cross-linking of KRP and smooth muscle myosin (44). KRP was shown to cross-link to both the RLC and the heavy chain and was localized to the neck region of myosin by electron microscopy. In addition, the cross-linked myosin was unable to form the 10 S conformation.

Recently Olney et al. (11) showed that myosin can be cross-linked in the 10 S conformation, using a probe on Cy506 of the RLC, to a site approximately one-third from the end of the tail. The cross-linked region on LMM contains one of the negatively charged regions within the 28-residue repeats, which characterize the myosin tail. It is interesting that the KRP sequence that we have identified to be most important for binding to myosin is also very acidic. We propose that the negatively charged carboxyl terminus of KRP may bind electrostatically to the same region of myosin to which the acidic tail interacts during 10 S formation and effectively compete with this tail to prevent 10 S formation.

In summary, our studies have shown that KRP binds tightly via its acidic tail to the site on myosin where S1 and S2 join. This binding site is logical based on KRP’s ability to affect the 6 S–10 S myosin equilibrium, and lends credence to KRP’s supposed in vivo function. Furthermore, implications that the 10 S conformation is related to ionic interactions between LMM and the S1-S2 junction correlate well with our finding that KRP binds to myosin via acidic residues. Further studies are currently under way to elucidate the specific residues of myosin and KRP that interact.

Acknowledgments—We thank Dr. Fei Wang for providing us with soluble myosin fragments, Dr. Henry Fales for performing mass spectrometry on KRP truncations, and Estelle Harvey and William Anderson for technical assistance. In addition, we are grateful to Dr. Robert S. Adelstein and Dr. Christine Cremo for their critical reading of the manuscript, and members of the Laboratory of Molecular Cardiology for advice and support.

REFERENCES

1. Somlyo, A. V., Butler, T. M., Bond, M., and Somlyo, A. P. (1981) Nature 294, 567–569
2. Murphy, R. A. (1994) FASEB J. 8, 311–318
3. Sellers, J. R., and Adelstein, R. S. (1987) in The Enzymes (Boyer, P. D. ed) pp. 381–418, Academic Press, Orlando
4. Trybus, K. M., and Lowey, S. (1984) J. Biol. Chem. 259, 8564–8571
5. Craig, R., Smith, R., and Kendrick-Jones, J. (1983) Nature 302, 436–439
6. Trybus, K. M., Huiatt, T. W., and Lowey, S. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 6151–6155
7. Ikere, M., Hinkins, S., and Hartsdige, D. J. (1983) Biochemistry 22, 4580–4587
8. Godfrain-DBecker, A., and Gillis, J. M. (1988) Adv. Exp. Med. Biol. 226, 149–154
9. Horowitz, A., Trybus, K. M., Bowman, D. S., and Fay, F. S. (1994) J. Cell Biol. 126, 1195–1200
10. Shirinsky, V. P., Vorontzkov, A. V., Birukov, K. G., Nanaev, A. K., Collinge, M., Lukas, T. J., Sellers, J. R., and Watterson, D. M. (1993) J. Biol. Chem. 268, 16578–16583
Interaction Sites between KRP and Myosin

25359

11. Olney, J. J., Sellers, J. R., and Cremo, C. R. (1996) J. Biol. Chem. 271, 20375–20384
12. Gallagher, P. J., and Herring, B. P. (1992) J. Biol. Chem. 267, 23945–23950
13. Gallagher, P. J., and Herring, B. P. (1992) J. Biol. Chem. 257, 13880–13883
14. Holden, H. M., Ito, M., Hartshorne, D. J., and Rayment, I. (1992) J. Biol. Chem. 267, 840–851
15. Vorotnikov, A. V., Silver, D. L., Sellers, J. R., Watterson, D. M., and Shirinsky, V. (1995) J. Muscle Res. Cell Motil. 17, 153a (abstr.)
16. Silver, D. L., Vorotnikov, A. V., Watterson, D. M., Shirinsky, V. P., and Sellers, J. R. (1996) Biophys. J. 70, A52 (abstr.)
17. Ito, M., Dabrowska, R., Guerriero, V., Jr., and Hartshorne, D. J. (1989) J. Biol. Chem. 264, 13971–13974
18. Sellers, J. R., and Pato, M. D. (1984) J. Biol. Chem. 259, 7740–7746
19. Sellers, J. R., Eisenberg, E., and Adelstein, R. S. (1982) J. Biol. Chem. 257, 13880–13883
20. Ikebe, M., and Hartshorne, D. J. (1985) Biochemistry 24, 2380–2387
21. Sugden, P. H., Holladay, L. A., Reimann, E. R., and Corbin, J. D. (1976) Biochem. J. 159, 409–422
22. Gill, S. C., and Von Hippel, P. H. (1989) Anal. Biochem. 182, 319–326
23. Laemmli, U. K. (1970) Nature 227, 680–685
24. Sellers, J. R., Sobeiro, M. S., Faust, K., Bengur, A. R., and Harvey, E. V. (1988) Biochemistry 27, 6977–6982
25. Olson, N. J., Pearson, R. B., Needleman, D. S., Hurwitz, M. Y., Kemp, B. E., and Means, A. R. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2284–2288
26. Herring, B. P., Gallagher, P. J., and Stull, J. T. (1992) J. Biol. Chem. 267, 25945–25950
27. Einheber, S., and Fischman, D. A. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2157–2161
28. Moss, C., Offer, G., Starr, R., and Bennett, P. (1975) J. Mol. Biol. 97, 1–9
29. Starr, R., and Offer, G. (1978) Biochem. J. 171, 813–816
30. Okagawa, T., Weber, F. E., Fischman, D. A., Vaughan, K. T., Mikawa, T., and Reina, F. C. (1993) J. Cell Biol 123, 619–626
31. Gilbert, R., Kelly, M. G., Mikawa, T., and Fischman, D. A. (1996) J. Cell Sci. 109, 101–111
32. Horwitz, R., Kempner, E. S., Bisher, M. E., and Podolsky, R. J. (1986) Nature 323, 160–164
33. Labeit, S., and Kolmerer, B. (1995) Science 270, 293–296
34. Herring, B. P., Gallagher, P. J., and Stull, J. T. (1992) J. Biol. Chem. 267, 25945–25950
35. Silver, D. L., Vorotnikov, A. V., Watterson, D. M., Shirinsky, V. P., and Sellers, J. R. (1996) Biophys. J. 70, A52 (abstr.)
36. Ito, M., Dabrowska, R., Guerriero, V., Jr., and Hartshorne, D. J. (1989) J. Biol. Chem. 264, 13971–13974
37. Benian, G. M., Tinley, T. L., Tang, X., and Borodovsky, M. (1996) J. Cell Biol. 132, 835–848
38. Kobe, B., Heierhorst, J., Feil, S. C., Parker, M. W., Benian, G. M., Weiss, K. R., and Kemp, B. E. (1996) EMBO J. 15, 6810–6821
39. Guerriero, V., Jr., Rowley, J. D., and Means, A. R. (1981) Cell 27, 449–458
40. Lin, P., Luby-Phelps, K., and Stull, J. T. (1997) J. Biol. Chem. 272, 7412–7420
41. Katoh, T., Kodama, T., Pakushima, A., Yazawa, M., and Morita, F. (1995) J. Biochem. (Tokyo) 118, 428–434
42. Onishi, H., and Watanabe, S. (1984) J. Biochem. (Tokyo) 95, 899–902
43. Ikebe, M., and Hartshorne, D. J. (1984) J. Biol. Chem. 259, 11639–11642
44. Masato, T., Numata, T., Katoh, T., Morita, F., and Yazawa, M. (1997) J. Biochem. (Tokyo) 121, 225–230