Communication

Restoration of Phosphorylation-dependent Regulation to the Skeletal Muscle Myosin Regulatory Light Chain*

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Regulation of the ATPase activity of smooth and nonmuscle myosin II involves reversible phosphorylation of the regulatory light chain (RLC). The RLC from skeletal muscle myosin (skRLC) is unable to confer regulation (myosin is locked in an inactive state) to smooth muscle myosin when substituted for the endogenous smooth RLC (smRLC). Studies of chimeric light chains comprised of the N- or C-terminal half of each skRLC and smRLC suggest that the structural basis for the loss of this regulation is within the C-terminal half of the RLC (Trybus, K. M., and Chatman, T. A. (1993) J. Biol. Chem. 268, 4412–4419). The purpose of this study is to delineate the structural elements within the C-terminal half of the smRLC that are absent in the skRLC and are necessary for regulation. By sequence comparison, six residues, Arg-103, Arg-123, Met-129, Gly-130, Arg-143, and Arg-160, which are conserved in regulated myosin RLCs but missing in nonregulated myosin RLCs, were identified in smRLC. To test whether these amino acids provide the missing structural elements necessary for phosphorylation-mediated regulation, a skRLC was engineered that replaced the corresponding skRLC amino acids (positions 100, 120, 126, 127, 140, and 157, respectively) with their smRLC counterparts. Using a newly developed RLC exchange procedure, the purified mutant protein was evaluated for its ability to regulate chicken gizzard smooth muscle myosin. Substitution of the six conserved amino acids into the skRLC completely restored phosphorylation-mediated regulation. Thus, a subset of these amino acids, including four basic arginine residues located in the E, F, G, and H helices (amino acids 127 and 126, respectively), may be the structural coordinates for the phosphorylation site in the N terminus. Based on this result, the regulation of glycogen phosphorylase is discussed as a model for the regulation of smooth muscle myosin.

Unlike striated muscle, the initiation of smooth muscle contraction must be preceded by serine phosphorylation of the myosin RLC which is accomplished by a calcium/calmodulin-dependent myosin light chain kinase in the presence of ATP (1). Although the RLC is also reversibly phosphorylated in vertebrate striated muscle (at a homologous serine), this phosphorylation simply modulates contractile activity (2). The loss of myosin regulation in striated muscle is due to undetermined alterations in the myosin heavy chain. Additionally, the smooth muscle and skeletal muscle RLCs are not functionally equivalent, even though the RLCs from skeletal muscle and smooth muscle are highly homologous (71% similarity and 53% identity in sequence). The RLC from skeletal muscle myosin (skRLC) is unable to confer regulation to smooth muscle myosin (a phosphorylation-regulated myosin) and locks the myosin in the "off" state when substituted for the endogenous smooth muscle RLC (smRLC) (3). Additionally, the smRLC can confer calcium sensitivity to scallop muscle myosin (Ca2+-regulated myosin), while the skRLC fails to do so (4). However, no RLC can inhibit the activity of vertebrate skeletal muscle myosin.

The fact that the skeletal muscle RLC has retained the structural elements to lock a phosphorylation-regulated myosin (smooth muscle myosin) into an inactive state that is not activated via RLC phosphorylation, presents an opportunity to use directed mutagenesis to gain insight into the mechanism of phosphorylation-dependent regulation. In the best understood example of phosphorylation-dependent enzyme regulation, that of glycogen phosphorylase (5), loss of ability to activate enzyme activity via phosphorylation could be achieved by loss of the two arginine residues that coordinate the phosphoserine. The purpose of this study was to ascertain if the loss of function of the skRLC could be restored by insertion of arginine residues that may function in a putative coordination of the phosphoserine.

The phosphorylated serine residue is near the N terminus of the RLC. Studies of chimeric RLCs comprised of the N- or C-terminal half of each skRLC and smRLC indicate that it is the C-terminal half of skRLC that lacks structural elements necessary for phosphorylation-mediated regulation (3). By sequence comparison (Fig. 1A), four arginine residues (Arg-103, Arg-123, Arg-143, and Arg-160; corresponding to amino acids 100, 120, 140, and 157, respectively, in the rabbit skRLC sequence) are present in the C-terminal half of the smRLC which are conserved in regulated myosin RLCs but missing in nonregulated myosin RLCs. The four arginines are located in four different domains (the E, F, G, and H helices) in the crystal structures of chicken skeletal myosin S1 (7) and the scallop myosin regulatory domain (8). In addition to these arginines, the C terminus of the skRLC was examined for other missing conserved amino acids. A striking substitution was for glycine 130 and methionine 129 (amino acids 127 and 126, respectively, in the rabbit skRLC) in the loop between the F and G helices, which is absolutely conserved in regulated myosins (Fig. 1A). The location of this glycine in the loop between two helices may confer flexibility that is necessary for the helices to translate relative to each other upon phosphorylation. This glycine provides a different function in Ca2+-regulation in scallop muscle (8). A functional role in regulation for

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1The abbreviations used are: RLC, regulatory light chain of myosin; smRLC, RLC from smooth muscle myosin; skRLC, RLC from skeletal muscle myosin; TFP, trifluoperazine.
MATERIALS AND METHODS

Preparation of Proteins—Dephosphorylated smooth muscle myosin was purified from either 35–70% or 40–60% ammonium sulfate fraction of chicken smooth muscle myosin as essentially described previously (12), except that the solubilized RLC proteins were purified by ion exchange chromatography on a DEAE-cellulose column (Whatman DE52).

Exchange of Wild Type and Mutant RLCs into Smooth Muscle Myosin—By using trifluoperazine (TFP), based on a RLC exchange and extraction procedure for skeletal muscle, a technique was developed that achieves a complete exchange of wild type and mutant skRLC into chicken smooth muscle myosin without elevating temperature (see Fig. 2). Chicken gizzard smooth muscle myosin was first incubated in an exchange solution containing 50 mM KCl, 10 mM EDTA, 10 mM CDTA, 5 mM ATP, 20 mM imidazole, 5 mM TFP, 0.05% Triton X-100, pH 7.0, in the presence of a 10 molar excess of the RLC to be exchanged. The exchange took place on ice with occasional shaking for 1.5 h. The myosin was then precipitated by adding 10 volumes of cold distilled H2O and pelleted by centrifugation at 4 °C. The pelleted myosin was dissolved in the exchange solution with a lowered (1.5 mM) TFP concentration in the presence of a 10 molar excess of the RLC to be exchanged. The exchange took place on ice with occasional shaking for 1.5 h. The myosin was then precipitated by adding 10 volumes of cold distilled H2O and pelleted by centrifugation. The extent of exchange was analyzed by SDS-PAGE gel electrophoresis.

Phosphorylation of RLCs—After the wild type and mutant skRLC and wild type smRLC were exchanged into chicken smooth muscle myosin, the RLCs were phosphorylated by incubating the hybrid myosin in 1 mM magnesium acetate, 50 mM Tris-HCl (pH 7.4), 0.25 mM CaCl2, 0.2 mM ATP, 0.05 mM EGTA, 5 μg/ml myosin light chain kinase (rat skeletal), and 1.6 μg/ml calmodulin for 10 min at 25 °C. The phosphorylation reaction was terminated by adding 1 mM EGTA to inhibit myosin light chain kinase. Under these conditions, the RLC was completely phosphorylated, as assessed by isoelectric focusing (10).

ATPase Assays—MgATPase activities of the wild type or mutant RLC-reconstituted chicken gizzard myosin were assayed under the following conditions: 30 mM KCl, 5 mM MgCl2, 1 mM ATP, 0.1 mM EGTA, 15 mM Tris-HCl (pH 7.6), 30–120 μg/ml myosin at 25 °C. Actin-activated MgATPase activities were assayed under the same conditions in the presence of 150–600 μg/ml actin purified from skeletal muscle. Actin-activated ATPase activities were calculated by subtracting the ATPase activities of myosin alone from the total actin-activated activity. Aliquots were removed at multiple time points to ascertain the linearity of phosphate release. Inorganic phosphate release was determined by a Malachite green-based colorimetric method, modified from published methods (13–15) as described previously (16).

RESULTS AND DISCUSSION

Exchange of RLCs into Chicken Smooth Muscle Myosin—The exchange of exogenous RLCs into myosin has proven difficult. For smooth muscle myosin, a 90% exchange of wild type smooth muscle RLC can be obtained at 42 °C in the presence of ATP and EDTA, provided that a 10-fold molar excess of wild type smooth RLC is present (3). Lower temperatures result in significantly less exchange of the RLCs. The skRLC has a lower affinity for the smooth muscle myosin heavy chain than the smRLC. Under the same conditions, 80% exchange requires a 70–80-fold molar excess of skRLC (3). By using trifluoperazine (TFP), near-complete exchange of skRLC into smooth muscle myosin was achieved on ice. A 10-fold molar excess of skeletal RLC was required to give a near-complete exchange.

Trifluoperazine is a calmodulin antagonist which inhibits the functions of calmodulin by formation of antagonist-calmodulin complex. The crystal structure of this complex in the presence of Ca2+ indicates that the binding of TFP induces conformational changes from an elongated dumbbell, with exposed hydrophobic surfaces, to a compact globular form which
can no longer interact with its target enzymes (17, 18). This conformational change is similar to that seen in the calmodulin-target peptide complex. Although the binding of TFP to RLC is not characterized, it is likely that a similar mechanism applies to TFP-RLC interactions. TFP may compete with the myosin heavy chain for the hydrophobic pockets of the RLC, thereby dissociating the RLCs. Previously, TFP has been used to extract troponin C (another member of the calmodulin superfamily) from the troponin complex (19) and to prepare RLC-deficient smooth muscle myosins through fast protein liquid chromatography gel filtration (3).

At a higher concentration (5 μm), TFP dissociates endogenous RLCs, but also interferes with the subsequent association of exogenous RLC. Presumably at high concentrations, TFP may effectively sequester the RLCs and prevent binding to the myosin heavy chain. On the other hand, lower TFP concentrations lead to a lower degree of exchange even at higher temperature (37°C), but does not influence the binding of the exogenous RLC. Based on these observations, the two-step RLC exchange procedure that involves lowering of the TFP concentration (described in above) was developed.

Regulation via Phosphorylation of the Myosins Containing Wild Type Smooth, Wild Type Skeletal, or the Mutant Skeletal RLC—Fig. 3 shows the actin-activated ATPase activities at 25°C of the dephosphorylated and phosphorylated chicken gizzard smooth muscle myosin. The endogenous RLC was exchanged for either chicken smooth muscle type RLC, rabbit skeletal wild type RLC, or the mutant skeletal RLC. The wild type smooth RLC-reconstituted gizzard myosin displays phosphorylation-mediated regulation. The actin-activated ATPase activity increases from 2 nmol/min/mg when wild type smRLC is dephosphorylated to 20 nmol/min/mg when the wild type smRLC is phosphorylated. These activities are comparable to what was found for native chicken gizzard myosin (22 nmol/min/mg at saturating levels of phosphorylation) (Fig. 3). The failure of the skeletal wild type RLC to confer the regulation of actin-activated ATPase via phosphorylation confirms previous findings (3). However, when the six conserved residues, Arg-103, Arg-123, Met-129, Gly-130, Arg-143, Arg-160, in smRLC replaced the corresponding residues of skRLC, the mutant skRLC fully restored phosphorylation-mediated regulation of gizzard myosin.

If phosphorylation of the RLC regulates myosin activity via a mechanism similar to that for the regulation of glycogen phosphorylase (5), then it is likely that of the labeled residues in Fig. 1B, two of the arginines are required to provide structural coordinates for the phosphoserine. When the phosphorylatable N-terminal serine is phosphorylated, the mobile and disordered N terminus may become immobilized and ordered and bind to the surface of the interface of the C-terminal domain. Based on the orientation of the corresponding residues in chicken skeletal structure (Fig. 1B), only the arginines found in helices E (Arg-100) and H (Arg-157) would appear to be positioned appropriately. One or two of the engineered arginines probably coordinates the phospho-phosphate of the phosphoryserine by hydrogen bonds. The coordination of the phosphoryserine may cause winding or unwinding of the helices and the rearrangement of domains. These conformational changes likely lead to regulation of myosin through altered interactions that involve the two RLCs and the myosin heavy chain.

Based on this model, one would predict that loss of the conserved arginines in the E and H helices of the smooth muscle RLC would result in a loss of regulation. Indeed, when Ikekber et al. (20) substituted a region of the skeletal RLC H helix into the smooth RLC H helix, Arg-157 was lost and so was regulation. Furthermore, again based on considering the regulation of glycogen phosphorylase, the N-terminal basic residues may be involved in stabilizing the interaction of the phosphoserine with its coordination sites. Such a mechanism would explain the results of the study of Ikekber and Morita (21), wherein cleavage of the smooth RLC removed Arg-13 and Arg-16 and generated a RLC that could not activate smooth muscle myosin (heavy meromyosin) even when serine 19 was phosphorylated. This hypothetical regulatory scheme also is supported by NMR results. The study of Levine et al. (22) suggests that the N-terminal region of both rabbit skRLC and gizzard smRLC exhibits segmental mobility independent of the rest of the molecule. When the RLC is phosphorylated, mobility of the N-terminal segment is diminished and the serine phosphate is influenced by neighboring positively charged side chains.

While the skeletal muscle RLC has maintained the ability to inhibit the activity of smooth muscle myosin, regulation (i.e. activation) of activity via phosphorylation of the RLC has been lost. Restoration of regulation results from the alteration of maximally six amino acids, and likely from a subset of the six, that are conserved in regulated myosins but absent in the vertebrate striated muscle RLCs. Additional mutagenesis, involving both the skeletal and smooth RLCs, will further delineate the necessary amino acids and the critical interactions involved in myosin regulation via phosphorylation. The regulation of glycogen phosphorylase should provide a useful framework for understanding the structural changes that accompany phosphorylation of the myosin RLC and which underlie the regulatory process.

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