Basic Residues Are Important for Activation but Not Autoinhibition of Rabbit Skeletal Muscle Myosin Light Chain Kinase*

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Several allosterically modulated protein kinases have been shown to be regulated by an autoinhibitory domain located within the kinase molecules. The inhibitory domain has been proposed to act as a "pseudosubstrate" inhibitor binding to the substrate binding site of the kinase, thereby blocking the binding of the enzyme's true substrate. In this report, site-directed mutagenesis has been used to further investigate the mechanism of activation of the inhibitory domain of rabbit skeletal muscle myosin light chain kinase. Basic residues within the pseudosubstrate domain (572-573, 577-579, 580-581), which are analogous to the important substrate determinants of the myosin light chain, were found not to be required in order to maintain the kinase in an inhibited state. Two groups of these residues (577-579 and 581-582) were, however, found to be important for high affinity calmodulin binding to the kinase. These data suggest that the autoinhibitory domain of myosin light chain kinase may not function by directly mimicking the light chain substrate.

Myosin light chain kinase is a Ca$^{2+}$/calmodulin-dependent protein kinase which specifically phosphorylates the regulatory light chain of myosin. Molecular studies have demonstrated that there are at least two distinct genes encoding different tissue-specific forms of the enzyme; one encodes the striated muscle form (Roush et al., 1988; Herring et al., 1989) and one the smooth and nonmuscle isoforms (Shoemaker et al., 1990). Hydrodynamic and CD spectroscopic data have been used to define a shape model for the rabbit skeletal muscle enzyme (Mayr and Heilmeyer, 1983). In this model the catalytic and regulatory domains, located at the center and carboxyl terminus, respectively, form a globular region, and the remaining amino-terminal portion is asymmetric and may form an extended rod-like structure. Comparison of the primary sequence of the rabbit skeletal muscle myosin light chain kinase with other protein kinases suggests that the catalytic core of the enzyme comprises residues 302-539 (Hanks et al., 1988). In addition it has been proposed that residues amino-terminal of this region (glutamic acids 269 and 270) are involved in protein substrate binding to the kinase (Herring et al., 1990b).

Limited chymotryptic digestion of rabbit skeletal muscle myosin light chain kinase results in the formation of a 35-kDa fragment (residues 256-584) which is catalytically active (Edelman et al., 1985), thus confirming the location of the catalytic domain. This chymotryptic fragment no longer requires calmodulin for kinase activity and does not bind to a calmodulin affinity column. In contrast, a tryptic fragment comprising residues 236-594 remains dependent on calmodulin for enzyme activity. These data together with synthetic peptide studies (Blumenthal and Krebs, 1987) have led to residues 577-593 being assigned as the calmodulin binding site of the enzyme preventing it from interacting with the light chain substrate (Kennelly et al., 1987). When Ca$^{2+}$/calmodulin binds to the kinase a conformational change occurs in which the inhibitory region is removed from the active site, thus reversing its inhibition of enzyme activity. This mechanism of regulation by a "pseudosubstrate" inhibitory domain has been proposed to be a general mechanism employed by all myosin light chain kinases and several other protein kinases (Pearson et al., 1988; Soderling, 1990). Recent studies on the chicken nonmuscle myosin light chain kinase have, however, suggested that the inhibitory region of that enzyme may not simply function as a pseudosubstrate inhibitor (Shoemaker et al., 1990). In the current study the mechanism of action of the inhibitory domain of the rabbit skeletal muscle myosin light chain kinase has been investigated. Using site-directed mutagenesis I have shown that basic residues within the proposed inhibitory domain are not required to maintain the kinase in an inhibited state in the absence of Ca$^{2+}$/calmodulin. In contrast, these residues are important for the high affinity binding of calmodulin to the kinase.

**MATERIALS AND METHODS**

**Protein Purification and Kinase Assays**—Rabbit skeletal muscle myosin light chain kinase was prepared as described previously (Nunnally et al., 1985) except that the Affi-Gel Blue column was replaced by a phenyl-Sepharose column (50 ml equilibrated with 10 mM MOPS, 7.0, 0.5 mM EDTA, 250 mM NaCl, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride). The kinase eluted in the flow-through fraction of this column. Rabbit skeletal muscle myosin light chains were purified according to Blumenthal and Stull (1980). Calmodulin was purified from bovine testes (Blumenthal and Stull, 1982). COS cell lysates were prepared by detergent lysis (1% Nonidet P-40). The abbreviations used are: MOPS, 4-morpholinepropanesulfonic acid; EGTA, ethyleneglycol bis(oxyethylene-nitriilo)tetraacetic acid.

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Expression and Ca\(^{2+}\)/Calmodulin Affinity of Mutant Kinases—Immunoblot analysis of COS cell lysates using anti-rabbit skeletal muscle myosin light chain kinase polyclonal antibodies demonstrates that all mutant myosin light chain kinases were expressed at levels similar to that of the wild type kinase with the exception of the double mutant PS2/3 which was expressed at 4–5-fold lower levels (data not shown). Changes in the Ca\(^{2+}\)/calmodulin affinity of the mutant kinases relative to the wild type enzyme was estimated by use of biotinylated calmodulin overlays of the kinases following immunoblotting using purified rabbit skeletal muscle myosin light chain kinase as a standard (Herring et al., 1990a). Ca\(^{2+}\)/calmodulin activation assays were performed as described by Blumenthal and Stull (1980). Assays were performed at a fixed high concentration of calmodulin (1.2 μM) and various concentrations of free Ca\(^{2+}\) as determined by Ca\(^{2+}\)/EGTA buffers (Potter and Gergely, 1975). The concentrations of other reactants are indicated in the figure and table legends.

Oligonucleotide-directed Mutagenesis—Oligonucleotide-directed mutagenesis was carried out by standard procedures (Sambrook et al., 1989). All mutations were confirmed by DNA sequencing. The following oligonucleotides were used to generate the PS series of altered myosin light chain kinases: 5'-GAGGTATTGCTGAAGCAAGTTTTGCTGCCAG-3' (PS1), 5'-TCCTCCAGGCTTCCATGAGGTTTCTTCCAGT-3' (PS2), 5'-ATGAAGTTTTGCTGCCAG-3' (PS3), 5'-GTTTTTCTTCCAGT-3' (PS4). The PS2/3 mutant was generated with PS2 as a template for the mutagenic PS3 oligonucleotide. The resultant amino acid substitutions generated are shown in Fig. 1.

Expression of Wild Type and Mutant Myosin Light Chain Kinases—Wild type and mutant cDNAs were subcloned into a pCMV 2 expression vector (Andersson et al., 1988) and expressed in COS cells as described previously (Herring et al., 1990a).

Immunoblot and Calmodulin Overlay Assays—Immunoblotting using polyclonal or monoclonal antibodies directed against rabbit skeletal muscle myosin light chain kinase was performed as described by Herring et al. (1990a). The same procedure was employed to generate blots which were reacted with biotinylated calmodulin (23 μg/ml; Billingsley et al., 1985) and developed using phosphatase-conjugated avidin, except that 10 mM CaCl\(_2\) was added to all solutions.

Expression and Ca\(^{2+}\)/Calmodulin Affinity of Mutant Kinases—Immunoblot analysis of COS cell lysates using anti-rabbit skeletal muscle myosin light chain kinase polyclonal antibodies demonstrates that all mutant myosin light chain kinases were expressed at levels similar to that of the wild type kinase with the exception of the double mutant PS2/3 which was expressed at 4–5-fold lower levels (data not shown). Changes in the Ca\(^{2+}\)/calmodulin affinity of the mutant kinases relative to the wild type enzyme was estimated by use of biotinylated calmodulin overlays of the kinases following transfer of the protein to nitrocellulose. Mutant kinases in which basic residues within the proposed calmodulin binding domain had been altered (PS2, -3, -4, and -2/3) no longer bound biotinylated calmodulin. In contrast, PS1, wild type kinase, and mutants of the proposed light chain binding region (LCB1, LCB2, and LCB3, Herring et al., 1990b) were readily detectable by this procedure (Fig. 2).

Kinetic Properties of PS Mutants—All the myosin light chain kinases produced by the PS series of mutations were completely dependent on Ca\(^{2+}\)/calmodulin for activity. Under standard assay conditions (i.e. at least a hundred-fold dilution of lysate into an assay) no kinase activity could be detected in the presence of 2 mM EGTA. When assayed at low dilution (10–20-fold) a small amount of \(^{32}\)P incorporation could be detected in the absence of calcium. The calcium-independent activity was less than 5% of total activity for all mutants except the double mutant PS2/3 (wild type, <0.1%; PS1, 0.3%; PS2, 2%; PS3, <0.1%; PS4, 2.9%; PS2/3, 6%). Assays performed under these conditions exhibit a significant background of \(^{32}\)P incorporation (approximately 0.1% of the total activity of the wild type enzyme) which was seen even in lysates produced from expression vectors in which the cDNA was oriented in the antisense direction (Herring et al., 1990a). The Ca\(^{2+}\)/calmodulin activation properties of wild type and mutant kinases were assessed by performing assays at a fixed high concentration of calmodulin and by using Ca\(^{2+}\)/EGTA buffers to alter the free calcium levels. Hence the Ca\(^{2+}\)/calmodulin concentration would be determined by the free Ca\(^{2+}\) concentration (“Materials and Methods”). Examples of the Ca\(^{2+}\) activation curves for the wild type kinase and the PS mutants are shown in Fig. 3. From these curves the concentrations of Ca\(^{2+}\) required to produce half-maximal activation of the enzymes were determined; these data are summarized in Table I. Alteration of basic residues amino-terminal of the proposed calmodulin-binding domain of myosin light chain kinase did not significantly change the concentration of Ca\(^{2+}\) required to half-maximally activate the enzyme (wild type [Ca\(^{2+}\)]\(_{0.5}\)max 0.12 μM; PS1 0.15 μM; Fig. 3, Table I). In contrast, altering basic amino acids within the proposed calmodulin binding domain of the kinase resulted

Fig. 2. Biotinylated calmodulin overlay of expressed myosin light chain kinases. Crude cell lysates containing 40 ng of expressed kinase (as determined by immunoblotting using monoclonal antibody 14a (Nunnally et al., 1987)) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose, and the blot was treated as described previously for monoclonal antibody (19a) immunoblots except that all solutions contained 10 mM CaCl\(_2\) (Herring et al., 1990a). In place of the first-step antibody 23 μg/ml biotinylated calmodulin was used, and avidin conjugated to alkaline phosphatase was used in place of a second-step antibody. The positions of molecular weight markers are shown to the left of the blot. Lane 1, 9 μl of lysate from cells transfected with the myosin light chain kinase cDNA in an antisense orientation; lanes 2–11, lysates (3–9 μl) containing 40 ng of the following myosin light chain kinases; lane 2, wild type; lane 3, LCB1; lane 4, LCB2; lane 5, LCB3; lane 6, PS1; lane 7, PS2; lane 8, PS4; lane 9, PS3. Lane 10 is a blank lane. Lane 11, 40 ng of PS2/3 lysate (30 μl). Mutant kinases LCB1, LCB2, and LCB3 were described previously (Herring et al., 1990b). All other mutations are as described under Fig. 1 and under “Materials and Methods.”
in a significant \( (p < 0.01, \text{paired t test}) \) increase in the concentration of \( \text{Ca}^{2+} \) required to half-maximally activate the enzymes (PS2, 0.336 \( \mu \text{M} \); PS3, 0.665 \( \mu \text{M} \); PS4, 2.41 \( \mu \text{M} \); PS2/3, 0.983 \( \mu \text{M} \); see Fig. 3 and Table I). In addition mutants PS3, PS4, and PS2/3 displayed significant \( (p < 0.01) \) reduction in their maximal kinase activities (see Fig. 3 and Table I). The \( K_m \) values of mutants PS3 and PS4 for myosin light chain and ATP were similar to those of the wild type enzyme (Table I).

**DISCUSSION**

Altering residues involved in maintaining myosin light chain kinase in an inactive state should result in a kinase which is more easily activatable or constitutively active. It has been shown that two groups of basic residues amino-terminal of the phosphorylatable serine of rabbit skeletal muscle myosin light chain are important substrate determinants for the kinase (Michnoff et al., 1986; Fig. 1). Thus, the pseudosubstrate hypothesis predicts that altering basic residues within the inhibitory domain would produce a less inhibited or constitutively active enzyme. In contrast, the results presented in this report demonstrate that alteration of these basic residues (577–579 and 581–582) produced mutant kinases which not only remained dependent on \( \text{Ca}^{2+}/\text{calmodulin} \) for activity but also required higher concentrations of \( \text{Ca}^{2+}/\text{calmodulin} \) to produce half-maximal activation than the wild type enzyme. In addition, the inability of mutant kinases PS3, PS4, and PS2/3 to be fully activated, even at very high \( \text{Ca}^{2+}/\text{calmodulin} \) concentrations, may suggest that the basic residues are important for the activation rather than the inhibition of kinase activity.

The increased \( \text{Ca}^{2+}/\text{calmodulin} \) concentrations required to activate mutant kinases PS2, PS3, PS4, and PS2/3 was due, at least in part, to a decreased affinity of the mutant kinases for \( \text{Ca}^{2+}/\text{calmodulin} \). This demonstrates the importance of these residues in the binding of the kinase to \( \text{Ca}^{2+}/\text{calmodulin} \) and is consistent with their location within the previously defined calmodulin binding domain of the enzyme. The greater concentration of \( \text{Ca}^{2+}/\text{calmodulin} \) required to activate PS4 (KRR → EEE) relative to PS2 (KRR → ETL) suggests that the introduced negative charges may be interacting with similarly charged residues in calmodulin. This proposal is in agreement with previous data in which similar mutations in an analogous position of the nonmuscle myosin light chain kinase were able to compliment reciprocal charge mutations in calmodulin (E84K, E120K; Shoemaker et al., 1990). Thus, it is reasonable to propose that the basic residues, at positions 577–579 and probably also those at positions 581–582, of the rabbit skeletal muscle myosin light chain kinase are interacting with acidic residues in calmodulin. It would, therefore, be difficult to envision that these same residues could be involved in the interaction of the inhibitory domain with the substrate binding site of the kinase. Nevertheless, it may be argued that mutation of the basic residues, within the inhibitory domain weakens, but does not obliterate, the interaction of the inhibitory region with the substrate binding site. Hence, the mutated kinases would remain \( \text{Ca}^{2+}/\text{calmodulin} \)-dependent and a potential decrease in the concentrations of \( \text{Ca}^{2+}/\text{calmodulin} \) required to half-maximally activate the kinases may be offset.

**Table I.** *Kinetic properties of mutant and wild type myosin light chain kinases***

| Kinase | \( K_m \) LC \( \mu \text{M} \) | \( K_m \) ATP \( \mu \text{M} \) | \( [\text{Ca}^{2+}]_{1/2 \text{max}} \) \( \mu \text{M} \) | \( V_{\text{max}} \) pmol/min/ng |
|--------|------|------|------------------|------------------|
| Purified | 5.0 | 244, 263 | 0.12 | 27.2 ± 4.1 (6) |
| WT | 3.5 ± 1.2 (4) | 336 ± 39 (4) | 0.12 ± 0.01 (3) | 33.6 ± 7.7 (10) 0% |
| PS1 | ND | ND | 0.15 ± 0.03 (5) | 35.6 ± 1.4 (5) 0.3% |
| PS2 | 6.9 | ND | 0.34 ± 0.06 (5) | 35.3 ± 2.6 (5) 2.0% |
| PS3 | 4.3, 3.6 | 278, 263 | 0.67 ± 0.06 (4) | 17.2 ± 0.6 (4) 0% |
| PS4 | 9.1, 4.8 | 400, 435 | 2.42 ± 0.10 (3) | 7.4 ± 1.2 (4) 2.9% |
| PS2/3 | ND | ND | 0.98 ± 0.06 (4) | 16.4 ± 1.5 (4) 6.0% |

*LC, light chain.

ND, not determined.
by their decreased affinity for Ca\(^{2+}\)/calmodulin. In support of this supposition a Ca\(^{2+}\)/calmodulin-independent form of the chicken nonmuscle myosin light chain kinase was produced by substitution of 6 basic residues, within the inhibitory region, with acidic glutamic acid residues (Shoemaker et al., 1990). However, the synthetic peptide studies of Michnoff et al. (1986) demonstrated a 40-50-fold increase in the \(K_m\) for peptides modeled after the myosin light chain, in which either group of important basic residues were replaced with the neutral amino acid alanine. If similar changes were to occur in the pseudosubstrate region mutant PS3 (KK → QQ 582-583) would be expected to exhibit a 50-fold decrease in affinity, and mutants PS2, PS4, and PS2/3 would exhibit a much greater decrease due to the the incorporation of acidic residues. Under the assay conditions used in this study, in which the light chain substrate is at 10-fold higher concentrations than its \(K_m\) value, one may have predicted that the substrate would be able to competitively overcome the inhibition exerted by the inhibitory domain.

Further evidence contradicting the importance of basic amino acids for the function of the inhibitory region can be obtained from proteolysis studies. A 35-kDa chymotryptic fragment of the rabbit skeletal muscle myosin light chain kinase (residues 256-584) is constitutively active (Edelman et al., 1985). This fragment still retains the basic residues analogous to the substrate determinants in the myosin light chain and would, therefore, according to the pseudosubstrate model, have been predicted to be inactive. In addition the inhibitory potency of synthetic peptides modeled after the inhibitory domain of smooth muscle myosin light chain kinase did not simply correlate with the number of basic residues in the peptide ( Lukas et al., 1988). These peptides have also been reported to inhibit enzyme activity competitively with respect to both ATP and myosin light chain ( Ikebe, 1990). Together these data suggest that the inhibitory region of myosin light chain kinase does not function by simply mimicking the light chain substrate. The data may be more consistent with a recently proposed “flip-flop” model describing the activation of calmodulin-dependent protein kinases (Jarrett and Madhavan, 1990).

In summary, the experiments presented here demonstrate that basic amino acids within the calmodulin binding domain of myosin light chain kinase are important for high affinity binding to Ca\(^{2+}\)/calmodulin. These residues may also be involved in the subsequent activation of the kinase. In contrast, no evidence was obtained to suggest that the basic residues are essential for the function of the inhibitory domain. This may indicate that the inhibitory region does not function by directly mimicking the myosin light chain substrate.

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