Properties of a Monoclonal Antibody Directed to the Calmodulin-Binding Domain of Rabbit Skeletal Muscle Myosin Light Chain Kinase†

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Received January 15, 1987; Revised Manuscript Received May 7, 1987

ABSTRACT: A synthetic peptide representing the calmodulin-binding domain of rabbit skeletal muscle myosin light chain kinase (K-R-R-W-K-K-N-F-I-A-V-S-A-A-N-R-F-K-K-I-S-S-S-G-A-L) was used as an antigen to produce a monoclonal antibody. The antibody (designated MAb $RSk_{CBP}1$, of the IgM class) reacted with similar affinity ($K_D \sim 20$ nM) by competitive enzyme-linked immunoassay (ELISA) with the antigen peptide and intact rabbit skeletal muscle myosin light chain kinase. MAb $RSk_{CBP}1$ inhibited rabbit skeletal muscle myosin light chain kinase activity competitively with respect to calmodulin ($K_i = 20$ nM). The antibody also inhibited myosin light chain kinase activity in extracts of skeletal muscle from several mammalian species (rabbit, sheep, and bovine) and an avian species (chicken). The concentration of MAb $RSK_{CBP}1$ required for 50% inhibition of enzyme activity was similar for the mammalian species (80 nM) but was significantly higher for the avian species (1.2 μM). A competitive ELISA protocol was used to analyze weak cross-reactivity to other calmodulin-binding peptides and proteins. This assay demonstrated no cross-reactivity with the venom peptides melittin or mastoparan; smooth muscle myosin light chain kinases from hog carotid, bovine trachea, or chicken gizzard; bovine brain calmodulin-dependent calcineurin; or rabbit skeletal muscle troponin I. These data support the contention that the synthetic peptide used as the antigen represents the calmodulin-binding domain of rabbit skeletal muscle myosin light chain kinase and that the calmodulin-binding domains of different calmodulin-regulated proteins may have distinct primary and/or higher order structures.

Myosin light chain kinase (ATPase:protein phosphotransferase, EC 2.7.1.37) is a well-characterized calmodulin-dependent enzyme that is found in both muscle and nonmuscle tissues. This protein kinase, which catalyzes the phosphorylation of a specific class of myosin light chains, occurs in tissue-specific forms that vary widely in molecular weight, antigenic properties, and catalytic properties [for review, see Stull et al. (1986)]. Phosphorylation of myosin by myosin light chain kinase occurs in vivo in both smooth and skeletal muscle. However, the physiological role of myosin phosphorylation in these two tissues is dissimilar. In smooth muscle, myosin phosphorylation appears to be essential for the initiation of contraction (Kamm & Stull, 1985), whereas in skeletal muscle myosin phosphorylation appears to play a modulatory role (Stull et al., 1985).

Monoclonal antibodies to rabbit skeletal muscle myosin light chain kinase have been generated to identify and characterize specific functional domains of this enzyme (Nunnally et al., 1987). Several of the monoclonal antibodies produced recognize epitopes located within the catalytic domain and inhibit myosin light chain kinase activity competitively with respect to the myosin P-light chain substrate. Inhibition of kinase activity by these antibodies was demonstrated to be noncompetitive with respect to calmodulin. Although monoclonal antibodies were produced that recognized several different domains of rabbit skeletal muscle myosin light chain kinase, none of the antibodies appeared to be directed to the calmodulin-binding domain.

Recently, the entire amino acid sequence of rabbit skeletal muscle myosin light chain kinase has been determined (Takio et al., 1985, 1986). The catalytic domain was shown to be located within the carboxyl half of the kinase (Edelman et al., 1985). A calmodulin-binding peptide of rabbit skeletal muscle myosin light chain kinase has also been identified and lies at the extreme carboxyl terminus of the kinase (Blumenthal et al., 1985).

Synthesis of a peptide representing the putative calmodulin-binding domain of rabbit skeletal muscle myosin light chain kinase (Blumenthal et al., 1985) has allowed us to use this synthetic peptide as an antigen to produce a monoclonal antibody to this important regulatory domain. A monoclonal antibody directed to the calmodulin-binding domain of rabbit skeletal muscle myosin light chain kinase has filled a gap in our library of antibodies directed to specific functional domains of the kinase. Additionally, this monoclonal antibody has been used to study the structural and evolutionary relationships between calmodulin-dependent enzymes, in particular the various forms of myosin light chain kinase. The characterization of this monoclonal antibody ($RSk_{CBP}1$)[1] with respect to its cross-reactivity to several calmodulin-binding peptides, myosin light chain kinases, and other calmodulin-dependent

†This research was supported, in part, by Grants HL23990 and HL26043 from the U.S. Public Health Service. Preliminary results of this study were presented at the annual meeting of the American Society of Biological Chemists, Washington, DC, June 1986.

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[1] Abbreviations: MAb, monoclonal antibody; RSk, rabbit skeletal muscle; CBP, calmodulin-binding peptide; ELISA, enzyme-linked immunoassay; BSA, bovine serum albumin; PBS, phosphate-buffered saline; MLCK, myosin light chain kinase.

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Table I: Amino Acid Sequences of Calmodulin-Binding Peptides and Proteins Tested for Cross-Reactivity with MAb $RSk_{CBP}1$

peptide or protein	sequence[a]	affinity[b]
M13 (26 residues)[c]	K-R-R-W-K-K-N-F-I-A-V-S-A-A-N-R-F-K-K-I-S-S-S-G-A-L	70 nM
MLCK4B (21 residues)[d]	K-R-R-R-W-K-K-A-F-I-A-V-S-A-A-A-R-F-K-K-I	25 nM
MLCK5 (18 residues)[e]	K-R-R-W-K-K-A-F-I-A-V-S-A-A-A-R-F-G-NH_2	>2 μM
melittin	G-I-G-A-V-I-L-K-V-L-T-T-G-L-P-A-L-I-S-W-I-K-R-K-Q-Q-NH_2	>9 μM
mastoparan	I-N-L-K-A-L-A-A-L-A-K-K-I-L-NH_2	>9 μM
gizzard MLCK[f]	R-R-K-W-Q-K-T-G-H-A-V-R-A-I-G-R-L-S-S-M-A-M-I-S-G	>2 μM

[a] The following one-letter codes are used to designate each amino acid: A (alanine), R (arginine), N (asparagine), Q (glutamine), G (glycine), H (histidine), I (isoleucine), L (leucine), K (lysine), F (phenylalanine), P (proline), S (serine), T (threonine), W (tryptophan), V (valine), and M (methionine). [b] Affinity is defined as the concentration of competitor required to achieve 50% inhibition as measured by competitive ELISA. For competitors where affinity is depicted as >2 μM, there was no inhibition even at concentrations listed. [c] M13 is a synthetic peptide based on the amino acid sequence of the rabbit skeletal muscle myosin light chain kinase calmodulin-binding domain. [d] MLCK4B is a synthetic peptide based on M13 containing an extra R and substituting A for N residues. [e] MLCK5 is a synthetic peptide based on M13 substituting A for N residues and containing a C-terminal glycylamide. [f] Sequence of putative calmodulin-binding domain for gizzard kinase from Lukas et al. (1986) and Guerriero et al. (1986).

proteins is described in this paper.

Experimental Procedures

Preparation of Protein Reagents. Myosin light chain kinase was purified from rabbit and chicken fast-twitch skeletal muscles as described previously (Nunnally et al., 1985). Smooth muscle myosin light chain kinases were purified from chicken gizzard, hog carotid, and bovine trachea muscles as described (Hathaway et al., 1985). Troponin–tropomyosin was purified from rabbit skeletal muscle as previously described (Stull & Buss, 1978). An approximately equimolar preparation of troponin I/troponin T was obtained by low-pH precipitation of tropomyosin and troponin C (Hartshorne & Mueller, 1968). Melittin and mastoparan were obtained from Sigma Chemical Co., St. Louis, MO. Synthetic peptides representing the calmodulin-binding domain of rabbit skeletal muscle myosin light chain kinase were synthesized as described by Blumenthal et al. (1985). Mice were immunized, with uncoupled peptide following standard immunization procedures, by use of a purified uncoupled synthetic peptide (Table I) representing the calmodulin-binding domain of the kinase. Monoclonal antibody production by fusion of immune mouse spleen cells to NS-1 myeloma cells was performed by standard procedures (Fazekas De St. Groth & Scheidegger, 1980). Antibody-producing hybridoma cells were injected intraperitoneally into mice for ascites fluid production, and the monoclonal antibody was purified from the ascites fluid by ammonium sulfate precipitation, followed by gel filtration chromatography. The purified monoclonal antibody (MAb $RSk_{CBP}1$) is of the IgM class (M_r 900 000). Protein and peptide concentrations were determined at 280 nm with the molar extinction coefficients (E) for rabbit skeletal muscle myosin light chain kinase (E = 39 000 M^{-1}) and the 21 and 26 amino acid residue peptides (E = 5555 M^{-1}). Other protein concentrations were determined by the Bradford assay (Bradford, 1976) with bovine serum albumin (BSA) (for other myosin light chain kinases) or immunoglobulin (for monoclonal antibody) as standards.

Competitive ELISA. Competitive binding assays to test antibody cross-reactivity to different peptides and proteins were based on a solid-phase, enzyme-linked assay (ELISA), which allowed for the detection of low-affinity interactions of the antibody with the test antigens. The assays were performed as follows. Polystyrene plates (96 well) were coated with 50 ng of rabbit skeletal muscle myosin light chain kinase overnight at 4 °C. The plates were incubated with phosphate-buffered saline containing 1 mg/mL BSA (PBS/BSA) for 1 h at 37 °C. Control wells contained 150 ng of MAb per 100 μL of PBS/BSA. Wells that contained competitor antigens contained 150 ng of MAb plus variable concentrations of competitor per 100 μL of PBS/BSA. Blank wells were included that contained no MAb and, therefore, served as background controls. The 96-well plate was then shaken on an orbital shaker for 30 min at room temperature, followed by a 2-h incubation, unshaken, at 37 °C. After incubation with the monoclonal antibody, the plate was washed 3 times with PBS + 0.05% Tween 20 and then incubated with alkaline phosphatase conjugated anti-mouse IgM antibodies (1 h at 37 °C). After this final incubation, the plate was washed as above and the alkaline phosphatase substrate added. The reaction was stopped at 10–20 min with 50 mM NaOH (50 μL). The volume of the wells was increased to 200 μL with water and the absorbance measured at 405 nm. Data were analyzed by subtracting the blank values from the control and experimental values and expressed as percent of control.

Antibody Inhibition of Kinase Activity. For measurements performed with purified kinases, assay conditions were as follows: 50 mM 3-(*N*-morpholino)propanesulfonic acid (pH 7.0), 10 mM magnesium acetate, 100 μM calcium chloride, 25 μM myosin P-light chain, calmodulin and antibody concentrations as indicated, and 0.3 nM myosin light chain kinase. Control tubes without antibody were included for each calmodulin concentration. The mixtures were incubated overnight at 4 °C and then for 10 min at 30 °C before [γ-^{32}P]ATP was added. After 2 and 4 min, aliquots were spotted on phosphocellulose paper, and the papers were washed in 75 mM phosphoric acid (Roskoski, 1983). Velocity values were calculated for each condition.

Skeletal muscle extracts were prepared as described previously (Nunnally & Stull, 1984) and diluted to yield comparable kinase activity in each tube (15–40 pmol of ^{32}P incorporated/min). The diluted extracts were preincubated with 0.5 nM calmodulin in the presence and absence of MAb. Preincubation prior to assay was performed at 4 °C for 2 h. Assays were performed by monitoring phosphorylation of myosin P-light chain as described above. Data are expressed as percent of control kinase activity (i.e., in the absence of MAb).

[γ-^{32}P]ATP was synthesized according to Walseth and Johnson (1979). ^{32}P was obtained from ICN, Irvine, CA.

Results

The binding of the monoclonal antibody (MAb $RSk_{CBP}1$) to the calmodulin-binding domain of rabbit skeletal muscle myosin light chain kinase was tested by ELISA against the peptide antigen. Additional preliminary characterizations included ELISA, immunoadsorption, and immunoblot analysis against purified rabbit skeletal muscle myosin light chain kinase (not shown). To compare the reactivity of MAb $RSk_{CBP}1$ to the peptide antigen relative to that of the intact

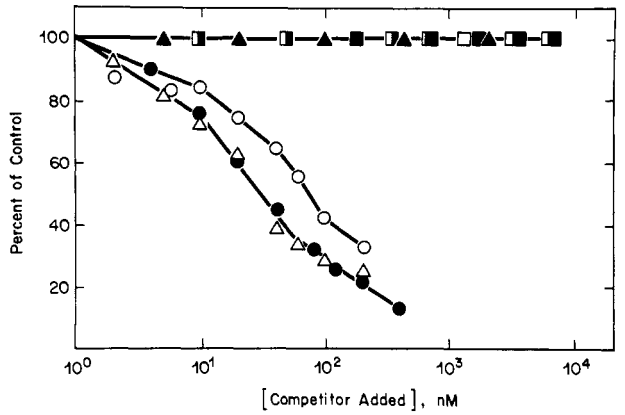

FIGURE 1: Monoclonal antibody cross-reactivity to several calmodulin-binding peptides. Competitor polypeptides RSkMLCK (●), M13 (○), MLCK4B (△), MLCK5 (▲), melittin (■), and mastoparan (□) were tested for cross-reactivity to MAb $RSk_{CBP}1$ by competitive ELISA. Data are plotted as percent of control vs. competitor concentration (nM).

kinase, a competitive ELISA protocol was designed. Figure 1 illustrates the reactivity of the antibody against the 26-residue peptide antigen (M13), two shorter peptides comprised of 18 and 21 residues (MLCK5 and 4B, respectively), and the intact kinase. Table I summarizes the cross-reactivity data of Figure 1 and shows the sequences of the peptides tested. MAb $RSk_{CBP}1$ shows similar affinity for the 26- and 21-residue calmodulin-binding peptides and the intact kinase. A shorter peptide (18 residues in length) did not react with MAb $RSk_{CBP}1$ at concentrations as high as 2 μM. This 18-residue peptide was, however, capable of binding calmodulin with high affinity as determined by competitive inhibition of kinase activity with respect to calmodulin (data not shown). Figure 1 also illustrates the lack of reactivity of MAb $RSk_{CBP}1$ with two unrelated calmodulin-binding peptides, melittin and mastoparan. Neither of these two peptides was capable of competing with the intact kinase for antibody binding at concentrations as high as 9 μM.

To determine if binding of MAb $RSk_{CBP}1$ to rabbit skeletal muscle myosin light chain kinase resulted in any alteration of kinase activity, the effect of antibody on kinase activity was tested in the absence and presence of calmodulin. There was no activation of kinase by antibody in the absence of calmodulin (data not shown). MAb $RSk_{CBP}1$ inhibited rabbit skeletal muscle myosin light chain kinase activity in the presence of 1 nM calmodulin, a concentration that results in half-maximal activation. To further analyze the nature of the antibody inhibition of kinase activity, the effect of calmodulin concentrations on inhibition of kinase activity by antibody was tested (Figure 2). The phosphorylation rate of rabbit skeletal muscle myosin light chain kinase was measured at varying calmodulin concentrations (1–20 nM) in the absence (control) and presence of 50 or 100 nM MAb $RSk_{CBP}1$. These data demonstrate that the inhibition of kinase activity by antibody was reversed by increasing calmodulin concentrations. Figure 3 displays a kinetic analysis of these data in a double-reciprocal plot of $(\text{velocity})^{-1}$ vs. $(\text{calmodulin concentration})^{-1}$. As the concentration of MAb $RSk_{CBP}1$ was increased from 50 to 100 nM, there was no change in the apparent V_{max} value, but there was a significant change in the K_{CaM} value relative to control (K_{CaM} is the calmodulin concentration required for half-maximal kinase activity at 100 μM Ca^{2+}). This pattern of inhibition is indicative of competitive inhibition by the antibody with respect to calmodulin. A replot of K_{CaM} and slope values vs. antibody concentration yields an apparent inhibition constant ($-K_i$) of the antibody with respect to calmodulin (K_i =

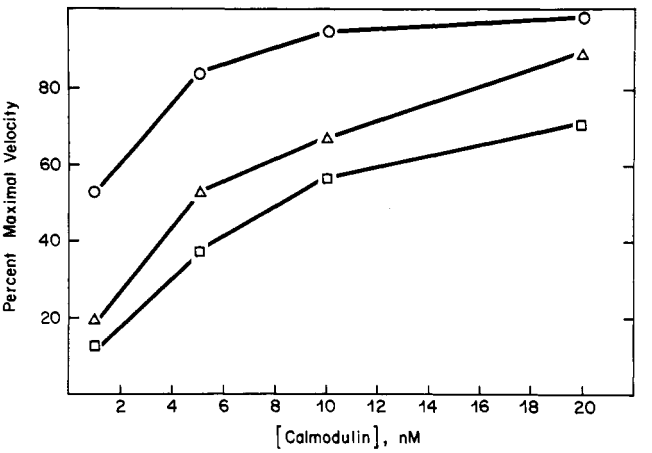

FIGURE 2: Effect of calmodulin on the antibody inhibition of rabbit skeletal muscle myosin light chain kinase activity. Rabbit skeletal muscle myosin light chain kinase was incubated as described under Experimental Procedures in the absence (○) or presence of 50 (△) or 100 nM (□) MAb $RSk_{CBP}1$. Data are expressed as percent maximal velocity vs. calmodulin concentration (nM).

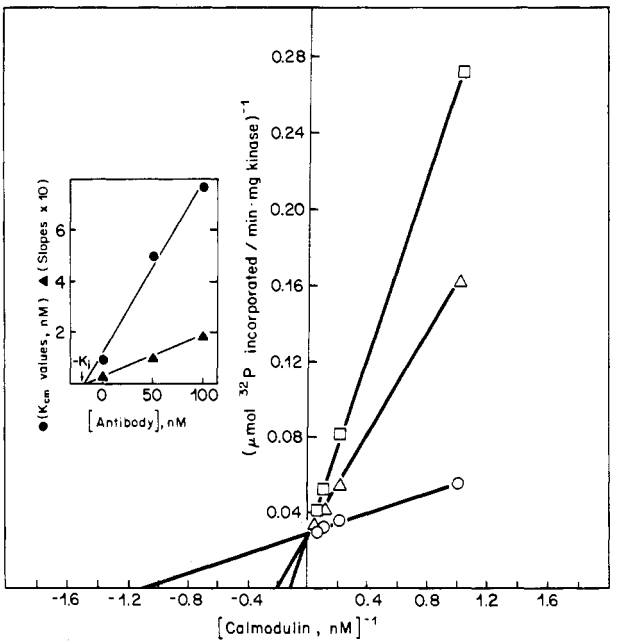

FIGURE 3: Double-reciprocal plot of antibody inhibition of myosin light chain kinase activity at varying calmodulin concentrations. Data from Figure 2 are replotted to yield a double-reciprocal plot [control (○); 50 nM MAb (△); 100 nM MAb (□)]. Inset depicts a replot of slopes (▲) and x intercepts (●) from the double-reciprocal plot vs. MAb $RSk_{CBP}1$ concentration (nM). The intercept of the replot yields the apparent inhibition constant ($-K_i$) of the antibody for inhibition of kinase activity with respect to calmodulin.

20 nM) (Figure 3, inset). Additionally, the linearity of the replot confirms that the inhibition is competitive with respect to calmodulin.

To determine if MAb $RSk_{CBP}1$ would show similar affinity to other skeletal muscle myosin light chain kinases, inhibition of kinase activity in extracts of skeletal muscles from several animal species was measured. Figure 4 illustrates the inhibition of kinase activity as a function of the monoclonal antibody concentration for extracts of rabbit, sheep, bovine, and chicken skeletal muscle. Inhibition of the kinase activity in the extracts of mammalian skeletal muscle showed very similar dependence on antibody concentration. The concentration of MAb $RSk_{CBP}1$ required to inhibit the various mammalian skeletal muscle kinase activities by 50% was approximately 80 nM. In contrast, approximately 15-fold more antibody (1.2 μM)

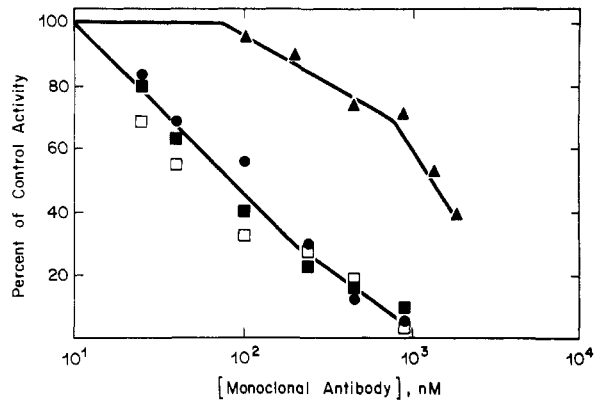

FIGURE 4: Antibody inhibition of myosin light chain kinase activity in extracts of mammalian and avian skeletal muscle. Extracts from rabbit (●), sheep (■), bovine (□), and chicken (▲) skeletal muscles were prepared and inhibition assays performed as described under Experimental Procedures. Data are expressed as percent of control activity vs. monoclonal antibody concentration (nM).

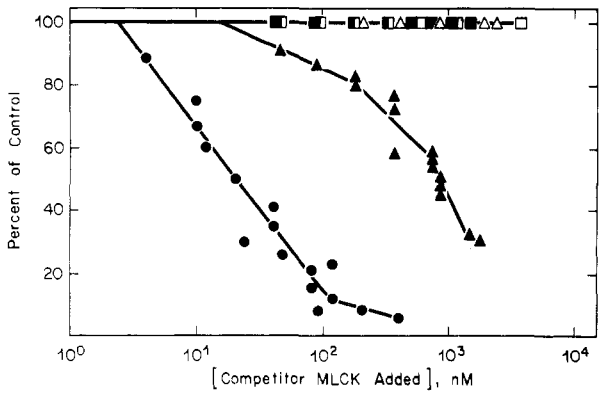

FIGURE 5: Antibody cross-reactivity to skeletal and smooth muscle myosin light chain kinases. Competitor kinases rabbit skeletal (●), chicken skeletal (▲), chicken gizzard smooth (△), hog carotid smooth (■), and bovine tracheal smooth (□) muscle myosin light chain kinases were tested for cross-reactivity to MAb $RSk_{CBP}1$ by competitive ELISA. Data are expressed as percent of control vs. competitor kinase concentration (nM).

was required to inhibit chicken skeletal muscle kinase activity to the same extent.

Figure 5 illustrates the relative affinity of MAb $RSk_{CBP}1$, measured by competitive ELISA, with purified rabbit skeletal, chicken skeletal, and several purified smooth muscle myosin light chain kinases (chicken gizzard, hog carotid, and bovine trachea). As demonstrated in earlier figures, MAb $RSk_{CBP}1$ had much greater affinity for rabbit skeletal muscle (apparent K_d = 20 nM) than for chicken skeletal muscle (apparent K_d = 1 μM) myosin light chain kinase. There was no cross-reactivity of the antibody with any of the smooth muscle myosin light chain kinases, either mammalian or avian. A similar analysis using bovine brain calcineurin (4 μM) and rabbit skeletal muscle troponin I (5 μM) as competitor proteins demonstrated no cross-reactivity with MAb $RSk_{CBP}1$ (data not shown).

Discussion

The data presented in this paper document that a monoclonal antibody prepared against a synthetic peptide representing the calmodulin-binding domain of rabbit skeletal muscle myosin light chain kinase reacts not only with the antigen peptide but also with the intact kinase. Cross-reactivity of an anti-peptide antibody with the intact protein from which the peptide sequence was derived is not unusual (Niman et al., 1983). It is, however, somewhat unusual that MAb $RSk_{CBP}1$ reacts with similar affinity to the antigen peptide and intact rabbit skeletal muscle myosin light chain kinase (Figure 1 and Table I). This similarity in affinity for either the peptide or intact kinase suggests that the antigen peptide and intact kinase have similar conformations or that it requires little loss of binding energy to fold either the peptide or enzyme into a conformation the MAb recognizes.

Like other calmodulin-binding peptides, the antigen peptide is an amphipathic structure with a high probability of α-helix formation. However, it has previously been shown by circular dichroism and proton nuclear magnetic resonance spectroscopy (Klevit et al., 1985) that, in aqueous solution, the calmodulin-binding domain peptide does not preferentially exist as an α-helix but that a substantial increase in α-helicity occurs upon the 1:1 binding of the peptide with Ca^{2+}–calmodulin. Taken together, these data suggest that, in the intact kinase, the calmodulin-binding domain of rabbit skeletal muscle myosin light chain kinase does not exist as an α-helix but that this conformation may be induced by the binding of Ca^{2+}–calmodulin. This suggestion is supported by the work of Mayr and Heilmeyer (1983), who demonstrated by circular dichroism spectroscopy an increase in α-helix content upon complex formation between calmodulin and either intact rabbit skeletal muscle myosin light chain kinase or large proteolytic fragments of the kinase. The sensitivity of this domain of the kinase to proteolysis also suggests that it does not exist as an α-helix but as a more extended structure (Edelman et al., 1985).

A shorter peptide representing part of the rabbit skeletal muscle myosin light chain kinase calmodulin-binding domain (MLCK5, Table I) does not react with MAb $RSk_{CBP}1$. These data indicate that the two lysines at positions 18 and 19, and perhaps the isoleucine at position 20, are very important determinants for binding affinity of MAb $RSk_{CBP}1$ to the peptide. Although it is possible that the antibody recognizes only these three residues on the calmodulin-binding peptide, this possibility seems unlikely because of the high affinity of the interaction. A more likely explanation is that these residues contribute significantly to the binding energy, perhaps by orienting the antigen to maximize a site of very strong interaction between the antibody and peptide antigen, or that these three amino acid residues are important to maintaining the secondary structure of the peptide that is recognized by the antibody. Clearly, further studies of peptide structure in solution correlated with antibody reactivity are necessary to address these questions.

It was anticipated that an antibody directed to the calmodulin-binding domain of myosin light chain kinase would, in some way, alter the activity of the kinase. One possibility is that antibody binding to the kinase would mimic Ca^{2+}–calmodulin binding, resulting in kinase activation. This has recently been observed with polyclonal antibodies reactive with the calmodulin-binding region of human erythrocyte plasma membrane Ca^{2+}-ATPase, which act as partial agonists, stimulating Ca^{2+}-ATPase activity and preventing further activation by calmodulin (Brandt et al., 1987). We tested this hypothesis by measuring kinase activity in the presence and absence of antibody at subsaturating calmodulin concentrations (Figure 2). There was no activation of kinase activity by MAb $RSk_{CBP}1$ in the absence of calmodulin; instead, the antibody was shown to inhibit kinase activity competitively with respect to calmodulin (Figures 2 and 3). These results provide further verification that the peptide used as antigen respresents the calmodulin-binding domain of rabbit skeletal muscle myosin light chain kinase.

The cross-reactivity of MAb $RSk_{CBP}1$ with other skeletal muscle myosin light chain kinases was tested by inhibition of kinase activity in extracts of skeletal muscle tissue from several other species (Figure 4). The antibody showed similar affinity for several mammalian skeletal muscle myosin light chain kinases (rabbit, bovine, and sheep) but significantly lower (15-fold) affinity for chicken skeletal muscle myosin light chain kinase. These data indicate that all of the mammalian skeletal muscle myosin light chain kinases must have calmodulin-binding domains of very similar sequence and/or structure. In contrast, the calmodulin-binding domain of the chicken skeletal muscle myosin light chain kinase must have a related but distinct structure.

Skeletal muscle and smooth muscle myosin light chain kinases appear to represent different forms of a related kinase (Nunnally et al., 1985). We were, therefore, interested to test MAb $RSk_{CBP}1$ against several purified smooth muscle myosin light chain kinases to determine if their calmodulin-binding domains are antigenically related to the calmodulin-binding domains in skeletal muscle myosin light chain kinases. Figure 5 depicts the data obtained by competitive ELISA with either purified skeletal muscle (rabbit and chicken) or smooth muscle (chicken gizzard, hog carotid, and bovine tracheal) myosin light chain kinases as competitors for antibody binding. The results demonstrate that none of the smooth muscle myosin light chain kinases were capable of binding to MAb $RSk_{CBP}1$ at concentrations as high as 4 μM. The lack of cross-reactivity of MAb $RSk_{CBP}1$ with the chicken gizzard smooth muscle myosin light chain kinase is not unexpected when the sequences of the calmodulin-binding domains of the rabbit skeletal and chicken gizzard smooth muscle kinases are compared (Table I). Although there are similarities in sequence, predominantly in the amino terminus, these two peptides do not show statistically significant identity (Blumenthal & Krebs, 1987). Additionally, MAb $RSk_{CBP}1$ requires the presence of residues 18–20 (K-K-I) for binding. These three amino acids are missing in the smooth muscle calmodulin-binding peptide. This difference alone may explain the nonreactivity of MAb $RSk_{CBP}1$ with gizzard kinase. Not only are the primary structures of the calmodulin-binding domains distinct between the rabbit skeletal and chicken gizzard smooth muscle myosin light chain kinases, but placement of these domains within the complete amino acid sequence of the two kinases is quite different. Blumenthal et al. (1985) demonstrated that the calmodulin-binding domain of rabbit skeletal muscle myosin light chain kinase represents the extreme carboxyl terminus of the kinase. In contrast, on the basis of the sequence of a partial cDNA clone of chicken gizzard smooth muscle myosin light chain kinase, Guerriero et al. (1986) demonstrated that the calmodulin-binding domain is located approximately 142 amino acids from the carboxyl terminus of the kinase. The work of Guerriero et al. (1986) corroborates the relative positions of the gizzard kinase catalytic and calmodulin-binding domains proposed by Foyt et al. (1985), as well as the amino acid sequence of the calmodulin-binding domain presented by Lukas et al. (1986). However, there are no published data on the placement or sequence of calmodulin-binding domains of any mammalian smooth muscle myosin light chain kinases, and therefore, no predictions as to the basis of MAb $RSk_{CBP}1$ non-cross-reactivity with mammalian smooth muscle myosin light chain kinases can be made. However, these results support our earlier contention that skeletal and smooth muscle myosin light chain kinases represent tissue-specific forms.

Recently, an affinity-purified anti-melittin antibody was shown to recognize the calmodulin-binding domains of calmodulin-dependent cAMP phosphodiesterase, chicken gizzard myosin light chain kinase, and eel electric organ $CaMBP_{55}$ (Kaetzel & Dedman, 1987). Using a competitive ELISA protocol, we tested MAb $RSk_{CBP}1$ for cross-reactivity against melittin and mastoparan, two calmodulin-binding peptides that have been used extensively in model studies of calmodulin–protein interactions (Malencik & Anderson, 1983; Maulet & Cox, 1983). There was no cross-reactivity of MAb $RSk_{CBP}1$ with these two calmodulin-binding polypeptides. Additionally, we demonstrated no cross-reactivity of MAb $RSk_{CBP}1$ against bovine brain calcineurin and rabbit skeletal muscle troponin I. These results, along with the lack of cross-reactivity with the smooth muscle myosin light chain kinases, demonstrate that MAb $RSk_{CBP}1$ is specific for skeletal muscle myosin light chain kinases.

In conclusion, our results suggest that although calmodulin-binding domains of proteins and model peptides may have generally similar properties with regard to overall probability of secondary structure and sequence composition, each type of calmodulin-binding protein may have sufficiently different primary structures to give rise to antigenically distinct calmodulin-binding domains.

ACKNOWLEDGMENTS

We acknowledge the expert technical assistance of Li-Chu Hsu for the preparation of the MAb $RSk_{CBP}1$ and subsequent help with experiments presented here. Bovine brain calcineurin was the generous gift of Dr. Claude Klee, NIH. We thank Kathy Perdue for assistance in the preparation of the manuscript.

Registry No. Synthetic peptide, 99268-57-2; myosin light chain kinase, 51845-53-5.

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A Physical–Chemical Model for Cellular Uptake of Fatty Acids: Prediction of Intracellular Pool Sizes†

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Received January 8, 1987; Revised Manuscript Received May 6, 1987

ABSTRACT: If the uptake of fatty acids by liver is a physical, not a biological, process, then the size and location of the intrahepatic pool of fatty acids can be predicted from uptake rates and thermodynamic data. The purpose of the experiments in this paper was to test the accuracy of this idea. Rat livers were perfused with palmitate bound to albumin, and the total amounts of palmitate removed from the perfusate were measured at 3-s intervals. The intrahepatic pools of palmitate calculated from these data were 13.8 and 23.0 nmol/g of liver at ratios of palmitate/albumin (mol/mol) (afferent side) of 2/1 and 4/1, respectively, in the steady state. The intrahepatic pools of palmitate calculated from the distributions of palmitate between membranes, H_2O, albumin, and fatty acid binding protein and the measured first-order rate constants for acyl-CoA ligases in mitochondria and microsomes were 12.1 and 34.6 nmol/g for perfusate ratios of palmitate/albumin of 2/1 and 4/1, in the steady state. Intrahepatic pools of palmitate measured after establishment of a steady-state rate of uptake were 15.0 and 31.8 nmol/g for these ratios of palmitate/albumin of 2/1 and 4/1.

Many water-insoluble compounds, such as long-chain fatty acids, bilirubin, and a number of drugs, circulate in blood bound tightly to albumin (Spector & Fletcher, 1978; Kamisaka et al., 1974). The mechanism for transfer of such compounds from albumin to the interiors of cells has been studied extensively (Spector et al., 1965; Spector & Steinberg, 1965; Mahadevan & Sauer, 1971, 1974; Samuel et al., 1976; DeGrella & Light, 1980; Abumrad et al., 1981, 1984; Stremmel et al., 1985; Weisiger et al., 1981a,b), but no consistent proposal for the mechanism has emerged. Results from early studies suggested that transfer of ligands from albumin to cellular membranes proceeded by simple diffusion (Spector et al., 1965; Spector & Steinberg, 1965). However, observations that uptake of fatty acids could be saturated (Abumrad et al., 1981, 1984; Stremmel et al., 1985; Weisiger et al., 1981a) and inhibited (Madhadevan & Sauer, 1971, 1974) were thought to be incompatible with a physical mechanism for uptake. This misconception (Noy et al., 1986) has led to the idea, now widely accepted, that uptake depends on a series of protein receptors (Abumrad et al., 1981, 1984; Madhadevan & Sauer, 1971, 1974; Samuel et al., 1976; Stremmel et al., 1985, 1986; Weisiger et al., 1981a). Moreover, the concept that hepatic uptake of compounds like fatty acids is mediated by a series of proteins fails to consider that these compounds are soluble in lipid bilayers, distribute readily from albumin to bilayers (Daniels et al., 1985), and rapidly traverse the latter (Deuticke, 1977; Doody et al., 1980; Eibl, 1984; Oldendorf, 1974; Noy & Zakim, 1985a; Noy et al., 1986).

As a first step in clarifying factors controlling the distribution of water-insoluble compounds in tissues and rates of uptake of these compounds into cells, we measured the spontaneous rates of the individual steps in the process of transfer of fatty acids from albumin to the interior of liver cells (Daniels et al., 1985; Noy & Zakim, 1985a; Noy et al., 1986). These observations provided a quantitative verification that the uptake of fatty acids, at least by liver, was a physical process (Noy et al., 1986). The purpose of the experiments in this paper was to test quantitative predictions about the size and intracellular locations of the intrahepatic pool of fatty acids, on the basis of the idea that transfer of fatty acids from al-

† This work was supported in part by the NIH (GM 33142). R.C. was supported by NIH Training Grant AM 07142.
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0006-2960/87/0426-5890$01.50/0 © 1987 American Chemical Society