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The γ-Subunit of Skeletal Muscle Phosphorylase Kinase Contains Two Noncontiguous Domains That Act in Concert to Bind Calmodulin*

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Phosphorylase kinase is a Ca^{2+}-regulated, multisubunit enzyme that contains calmodulin as an integral subunit (termed the δ-subunit). Ca^{2+}-dependent activity of the enzyme is thought to be regulated by direct interaction of the δ-subunit with the catalytic subunit (the γ-subunit) in the holoenzyme complex. In order to systematically search for putative calmodulin (δ-subunit)-binding domain(s) in the γ-subunit of phosphorylase kinase, a series of 18 overlapping peptides corresponding to the C terminus of the γ-subunit was chemically synthesized using a tea bag method. The calmodulin-binding activity of each peptide was tested for its ability to inhibit Ca^{2+}/calmodulin-dependent activation of myosin light chain kinase. Data were obtained indicating that two distinct regions in the γ-subunit, one spanning residues 287–331 (termed domain-N) and the other residues 332–371 (domain-C), are capable of binding calmodulin with nanomolar affinity. Peptides from both of these two domains also inhibited calmodulin-dependent reactivation of denatured γ-subunit. The interactions of peptides from both domain-N and domain-C with calmodulin were found to be Ca^{2+}-dependent. Dixon plots obtained using mixtures of peptides from domain-N and domain-C indicate that these two domains can bind simultaneously to a single molecule of calmodulin. Multiple contacts between the γ-subunit and calmodulin (δ-subunit), as indicated by our data, may help to explain why strongly denaturing conditions are required to dissociate these two subunits, whereas complexes of calmodulin with most other target enzymes can be readily dissociated by merely lowering Ca^{2+} to submicromolar concentrations. Comparison of the sequences of the two calmodulin-binding domains in the γ-subunit of phosphorylase kinase with corresponding regions in troponin I indicates similarities that may have functional and evolutionary significance.

Skeletal muscle phosphorylase kinase catalyzes the phosphorylation of glycogen phosphorylase and serves a key regulatory role in the process of glycogen metabolism (reviewed by Pickett-Gies and Walsh, Ref. 1). The enzyme has a molecular weight of 1.3×10^6 and has a subunit composition of $(\alpha\beta\gamma\delta)_4$. The γ-subunit, the catalytic subunit of this multisubunit protein kinase (2–4), is regulated by the other subunits of the holoenzyme in response to various physiological stimuli (1). The activity of phosphorylase kinase is Ca^{2+}-dependent, and this mode of regulation can be attributed to direct interactions between the γ-subunit and the δ-subunit, which is identical with calmodulin (5–9). Calmodulin is a ubiquitous Ca^{2+}-binding protein that modulates the activity of a wide variety of enzymes by reversibly binding to them in response to changes in intracellular calcium concentration. Phosphorylase kinase is different from most of the known calmodulin-regulated enzymes in that the δ-subunit stays tightly associated in the holoenzyme complex in the absence of Ca^{2+}. In addition to the δ-subunit, each molecule of the skeletal muscle form of phosphorylase kinase can reversibly bind four additional molecules of calmodulin (termed δ′-subunits) in a Ca^{2+}-dependent manner (10). The stimulation of catalytic activity by the δ′-subunit appears to be mediated through binding to the α- and β-subunits of the holoenzyme (10).

The amino acid (2) and nucleotide (11) sequences of the γ-subunit of rabbit skeletal muscle phosphorylase kinase have been determined, as well as nucleotide sequences from rat and mouse skeletal muscle (12–14). A cDNA sequence has also recently been reported which may represent a non-muscle form of the enzyme (15). The sequence spanning amino acid residues 20–276 in the skeletal muscle γ-subunit is homologous to the sequences of catalytic domains from other protein kinases, consistent with this region of the molecule being the catalytic domain (2). The C-terminal 110-residue sequence of the γ-subunit shows little homology to any other protein kinase sequence, suggesting that this region may play a role unique to phosphorylase kinase, such as in subunit-subunit interactions. In the present study, a systematic search of the C-terminal region of the γ-subunit (residues 276–386) was undertaken in order to identify possible calmodulin-binding domain(s). The search technique involves chemically synthesizing a series of overlapping peptides using a tea bag method (16) and assaying each of the peptides for its ability to bind calmodulin. This technique has general application to protein structure-function analysis and has also been recently used to study the domain structure of von Willebrand factor (17).

EXPERIMENTAL PROCEDURES

Synthesis and Purification of Synthetic Peptides—Peptides were synthesized by standard solid phase techniques using N^{α}-*t*-butoxycarbonyl amino acid derivatives, benzhydrylamine resin (Peninsula Laboratories and Chemical Dynamics), and a tea bag methodology (16) using a Biosearch SAM II automated solid phase peptide synthesizer modified for this purpose. The synthesis protocol was essentially as previously described (18). Peptides were cleaved from the resin and deprotected by treatment with anhydrous HF containing 10% anisole. Peptides containing histidine were also treated with β-mercaptoethanol before HF cleavage to remove the dinitrophenyl side chain protecting group. Peptides were purified by gel filtration (P2, Bio-Rad) followed by ion exchange chromatography using either

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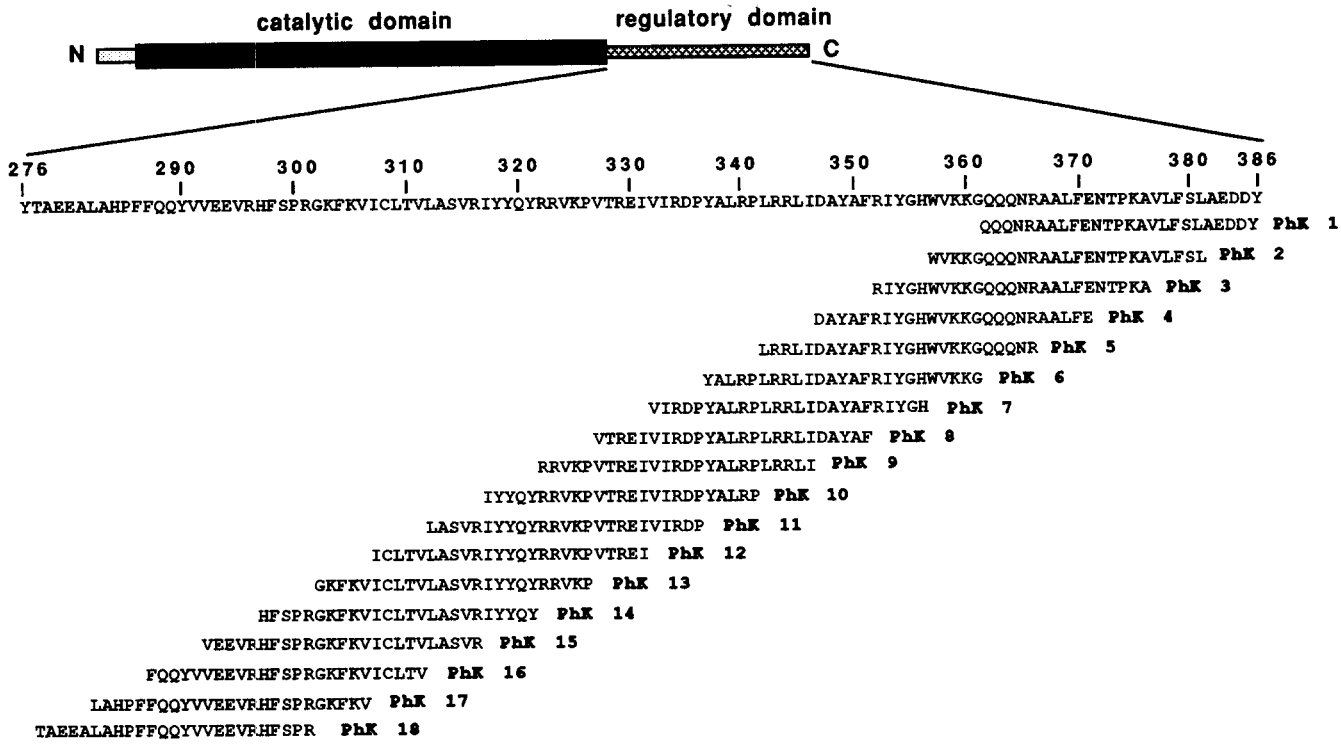

FIG. 1. **Nomenclature of synthetic γ-subunit (PhK) peptides.** The 18 overlapping 26-residue peptides shown were synthesized as described under "Experimental Procedures." Each peptide was assigned a *number* based on its position relative to the C terminus of the γ-subunit of phosphorylase kinase. The C-terminal Gly-amide residue present on each peptide is not shown. The sequence of each peptide is aligned with its corresponding sequence in the whole γ-subunit polypeptide.

DEAE- (Fractogel TSK DE-650M) or CM- (Fractogel TSK CM-650M) column chromatography, depending on the charge of the peptide. The peptides were finally purified by reversed phase HPLC[1] using a Vydac C-4 (5 μm, 300 Å pore) analytical column. The composition and sequence of each peptide was confirmed by using a Beckman Model 6800 amino acid analyzer and an Applied Biosystems Protein Sequencer Model 477A.

Protein Preparation—Rabbit skeletal muscle myosin light chain kinase was purified as described by Takio *et al.* (19). Calmodulin was prepared from bovine testis by using batchwise DEAE-cellulose (DE52, Whatman) chromatography, phenyl-Sepharose (Sigma) chromatography (20), and gel filtration chromatography (Bio-Gel A-0.5 m, Bio-Rad). Phosphorylase kinase was purified according to Cohen (21). Phosphorylase *b* was prepared according to Krebs and Fisher (22). The γ-subunit of phosphorylase kinase was prepared using reversed-phase HPLC as described by Crabb and Heilmeyer (23), except that a Vydac C-4 column was used instead of a C-18 column. An elution pattern similar to that reported for the C-18 column was obtained using the C-4 column.

Myosin Light Chain Kinase Assay—The rate of incorporation of ^{32}P into a synthetic peptide substrate (KKRPQRATSNVFS-amide) corresponding to the phosphorylation site of smooth muscle myosin P-light chain was measured. The reaction mixtures for inhibition experiments contained 50 mM MOPS, pH 7.0, 1 mM dithiothreitol, 130 μM substrate peptide, 10 mM magnesium acetate, 0.2 mM $CaCl_2$, 10 nM calmodulin, 1.3 nM skeletal muscle myosin light chain kinase, 1 mM [γ-^{32}P]ATP (≈300 cpm/pmol; Du Pont-New England Nuclear), and varying concentrations of synthetic γ-subunit peptides. The reaction was carried out at 30°C, and 20-μl samples of the reaction were stopped at two time points (5 and 15 min). Mixed inhibition experiments were done in the same way except that prereaction mixtures containing 100 μg/ml bovine serum albumin, γ-subunit peptides, and calmodulin (but without enzyme, substrate peptide, or [γ-^{32}P]ATP) were preincubated at room temperature overnight to allow the peptides and calmodulin to come to equilibrium.

Reactivation of γ-Subunit—Reactivation of the γ-subunit was performed based on the procedure described by Kee and Graves (4). Reactivation reaction mixtures contained 50 mM Tris, 50 mM β-glycerol phosphate, pH 8.0, 2 mM dithiothreitol, 3 μM calmodulin, 1 mg/ml bovine serum albumin, HPLC-purified γ-subunit diluted at least 10-fold (less than 1 μg/ml final), and 1 μM concentration of a given γ-subunit peptide. Reactivation was done in a 50-μl reaction volume at 0°C for 18 h. The extent of reactivation of the γ-subunit was measured by determining the rate of incorporation of ^{32}P into phosphorylase *b*. All reaction mixtures (50 μl) contained 50 mM Tris, 50 mM β-glycerol phosphate, pH 8.6, 10 mM magnesium acetate, 0.2 mM $CaCl_2$, 5 mg/ml phosphorylase *b*, 5 μl of the reactivation mixture, and 1 mM [γ-^{32}P]ATP (≈300 cpm/pmol). The reaction was carried out at 30°C, and 20-μl samples of the reaction were stopped at 5 and 15 min.

Mobility Shift Assay—The direct binding of the peptides to calmodulin was demonstrated by mobility shifts of calmodulin on electrophoresis in 10% polyacrylamide gels in the presence or absence of 4 M urea as described by Erickson-Viitanen and DeGrado (24).

Protein Estimation—The concentration of myosin light chain kinase was determined by the method of Bradford (25) using bovine serum albumin as a standard. Other protein concentrations were determined spectrophotometrically using values of $E_{280\ nm,10\ mg/ml}$ of 12.4 (21) and 13.2 (26) for phosphorylase kinase and phosphorylase *b*, respectively, and a value of $E_{276\ nm,10\ mg/ml}$ of 1.8 (27) for calmodulin. The γ-subunit concentration was determined as described by Kee and Graves (4). Concentrations of synthetic peptide stock solutions were determined by amino acid analysis.

Kinetic Analysis—The K_i values for the inhibitory peptides were determined using the following equation (28):

$$v_i = \frac{v_o}{\frac{K_{CaM}}{[CaM]}\left(1+\frac{[i]}{K_i}\right)+1} \quad (1)$$

where v_0 is the reaction rate at 10 nM calmodulin in the absence of any inhibitor, v_i is the reaction rate in the presence of a peptide inhibitor, $[i]$ is the inhibitor peptide concentration, K_{CaM} is the concentration of calmodulin required for half-maximal activation of

[1] The abbreviations used are: HPLC, high performance liquid chromatography; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; TnI, troponin I; TnC, troponin C; MOPS, 4-morpholinepropanesulfonic acid.

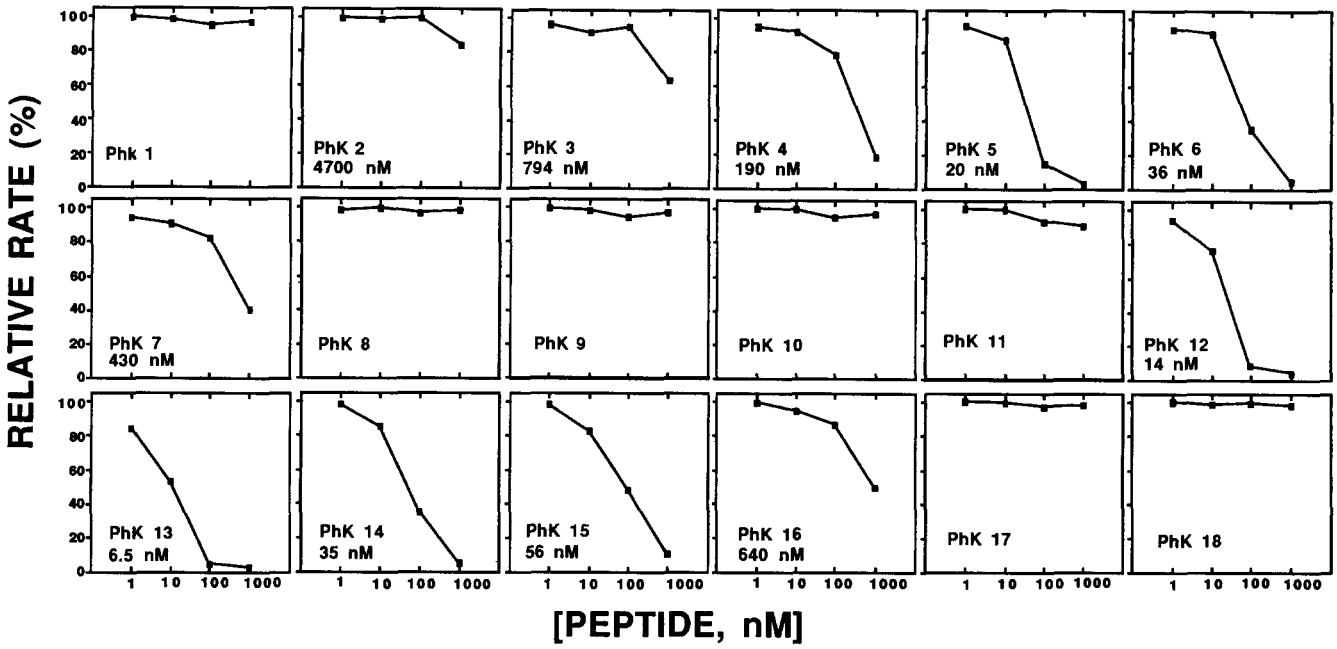

FIG. 2. **Concentration-dependent inhibition of calmodulin-dependent myosin light chain kinase activity by γ-subunit (PhK) peptides.** The 18 synthetic γ-subunit (PhK) peptides were tested for their ability to inhibit Ca^{2+}/calmodulin-dependent myosin light chain kinase activity. The details of the assay conditions are described under "Experimental Procedures." Peptides were used at the concentrations indicated on the *abscissa*. Catalytic rates are expressed relative to the reaction rate in the absence of any inhibitor and in the presence of 10 nM calmodulin. The apparent K_i value for each inhibitory peptide was calculated by using Equation 1 (see "Experimental Procedures") and is shown in each plot.

myosin light chain kinase (4 nM), and [CaM] is the calmodulin concentration used in the reaction mixture (10 nM).

The equation used to analyze data from the mixed inhibition experiments with PhK 13 and PhK 5 was (29):

$$\frac{v_o}{v_i} = \frac{K_{CaM}}{[CaM]\,K_z}\left(1+\frac{[X]}{\alpha K_x}\right)[Z] + \left(1+\frac{K_{CaM}}{[CaM]}+\frac{K_{CaM}\,[X]}{[CaM]\,K_x}\right) \quad (2)$$

where v_i is the reaction rate at $[X]$ and $[Z]$ concentrations of PhK 5 and PhK 13, respectively, K_x and K_z are the K_i values for PhK 5 and PhK 13, respectively, and α is the cooperativity factor between PhK 5 and PhK 13 with respect to their binding to calmodulin. The values of α, K_x, and K_z were determined by Marquardt weighted nonlinear least squares fit estimation using the Math View Professional program run on a Macintosh computer using the data points shown in Fig. 5. The values determined for K_x and K_z using Equation 2 were in good agreement with K_i values determined in separate experiments using Equation 1.

RESULTS

A series of 18 overlapping peptides (see Fig. 1) was synthesized corresponding to the C-terminal 110-residue sequence of the γ-subunit of phosphorylase kinase. The length of each peptide was 26 residues (including a C-terminal glycyl residue). A new peptide was initiated every 5th residue starting from the C terminus such that every 20-residue segment between residues 276 and 386 of the γ-subunit is represented in one of the synthesized peptides. The key assumption underlying this approach was that a 20-residue segment should have sufficient structure to permit identification of a calmodulin-binding domain since this is the case with most calmodulin-binding proteins that have been studied to date (18).

The ability to inhibit the Ca^{2+}/calmodulin-dependent activation of skeletal muscle myosin light chain kinase was used to quantitate the calmodulin-binding activity of each of the synthetic γ-subunit peptides (Fig. 2). Although about half of the peptides showed little or no inhibitory activity at concentrations as high as 1 μM, several of the peptides exhibited concentration-dependent inhibition of myosin light chain kinase activity. The apparent K_i values of the inhibitory peptides were calculated using Equation 1 (see "Experimental Procedures") and are presented in Fig. 2. For comparison, the K_i value obtained for the synthetic calmodulin-binding domain peptide of skeletal muscle myosin light chain kinase (KRRWKKAFIAVSAAARFG-amide, termed MLCK-5) was found to be 2.3 nM (data not shown), in good agreement with previously published values (30). Several of the γ-subunit peptides were found to bind calmodulin with reasonably high affinity as indicated by K_i values in the nanomolar range. The relative positions of the inhibitory and noninhibitory peptides within the overall sequence of the γ-subunit are shown in Fig. 3*A*. There appear to be two distinct domains in the C terminus of the γ-subunit that can interact with calmodulin. The region hereafter referred to as domain-N spans residues 287–331 and contains PhK peptides 12 to 16, whereas domain-C spans residues 332–371 and encompasses PhK peptides 4 to 7. The most inhibitory peptides in domain-N and domain-C were PhK 13 (K_i = 6.5 nM) and PhK 5 (K_i = 20 nM), respectively. The high affinity of these two peptides for calmodulin suggests that each may contain most, if not all, of the necessary calmodulin-binding determinants for that domain.

The γ-subunit peptides were also assayed for their ability to inhibit the refolding of denatured γ-subunit, a process that uses calmodulin as a template (4). Although peptide-calmodulin binding constants cannot be accurately estimated using the reactivation assay because of the high concentrations of calmodulin (3 μM) and peptides (1 μM) required and because the calmodulin dependence of γ-subunit reactivation does not follow simple hyperbolic kinetics,[2] the reactivation assay can provide semiquantitative estimates of the inhibitory capacity of each peptide. A pattern of inhibition very similar to that obtained with the myosin light chain kinase assay was ob-

[2] M. Dasgupta and D. K. Blumenthal, unpublished observations.

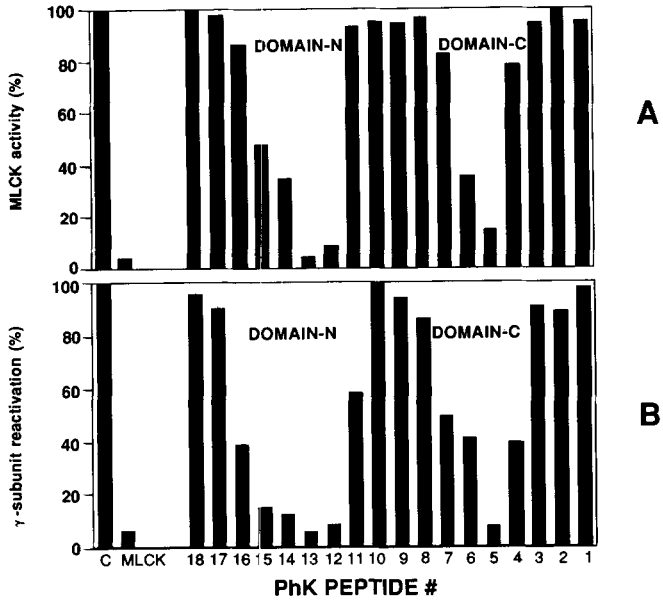

FIG. 3. **Relative inhibitory effect of γ-subunit (PhK) peptides on Ca^{2+}/calmodulin-dependent myosin light chain kinase activity and reactivation of γ-subunit.** *A*, enzyme activity of myosin light chain kinase was measured in the presence of the indicated synthetic γ-subunit (PhK) peptide (100 nM) and calmodulin (10 nM). Details of the assay conditions are given under "Experimental Procedures." Catalytic rates are expressed relative to the reaction rate in the absence of any peptide (column labeled *C*). The column denoted *MLCK* represents the activity in the presence of 100 nM MLCK-5 peptide. *B*, reactivation of HPLC-purified γ-subunit was determined in the presence of the indicated synthetic γ-subunit (PhK) peptide (1 μM) and calmodulin (3 μM). Isolation of γ-subunit and details of the reactivation conditions are described under "Experimental Procedures." Reactivation rates are expressed relative to those obtained in the absence of any peptide (labeled as *C*). The inhibitory effect of 1 μM MLCK-5 peptide on γ-subunit reactivation is shown in the column denoted *MLCK*.

tained for the reactivation assay (Fig. 3*B*). In the case of both assays, high concentrations of calmodulin could completely overcome the inhibitory activity of any peptide (data not shown) indicating that under the assay conditions used, peptide inhibition is competitive with respect to calmodulin and is not due to a direct effect of the peptides on the enzyme or other components in the reaction mixture (data not shown). Analyses of peptide inhibition using Dixon plots as described below were also consistent with a competitive mechanism of inhibition.

Because of their high affinity, the peptides denoted as PhK 13 and PhK 5 were selected as being representative of calmodulin-binding domain-N and domain-C, respectively. The remainder of the experiments described below are primarily restricted to these two peptides. A mobility shift assay was performed to determine whether the binding of either PhK 13 or PhK 5 to calmodulin is Ca^{2+}-dependent. In the presence of Ca^{2+} and 4 M urea, the electrophoretic mobility of calmodulin was found to be retarded by both peptides (Fig. 4*A*), indicating the formation of a complex between each of the peptides and Ca^{2+}/calmodulin. For reference, the mobility of calmodulin in the presence of the synthetic calmodulin-binding domain peptide of myosin light chain kinase, MLCK-5, is shown in Fig. 4*A*. Neither of the γ-subunit peptides formed a complex with calmodulin in the presence of the Ca^{2+} chelator, EGTA, indicating that both of the γ-subunit calmodulin-binding domain peptides require Ca^{2+} for binding to calmodulin. This is also the case for the MLCK-5 peptide (Fig. 4*A*). Electrophoretic mobility shift assays were also performed under nondenaturing conditions (Fig. 4*B*). Both of the γ-subunit peptides formed complexes with calmodulin in the presence of Ca^{2+}, but not in the presence of EGTA, indicating that 4 M urea is not required to demonstrate Ca^{2+}-dependent interaction of the peptides with calmodulin. By varying the ratio of peptide to calmodulin using the mobility shift assay, the approximate molar stoichiometry of both γ-subunit peptides for calmodulin was found to be 1:1 (data not shown).

The identification of two distinct calmodulin-binding domains in the γ-subunit suggested the possibility that the two domains can bind calmodulin simultaneously. To investigate this possibility, mixtures of various concentrations of PhK 13 and PhK 5 were added to the reaction and assayed for the ability to synergistically inhibit myosin light kinase activity. Dixon plots of v_0/v_i *versus* the concentration of PhK 13 at different fixed concentrations of PhK 5 are shown in Fig. 5*A*. The same data are plotted in Fig. 5*B*, but as a function of PhK 5 at different concentrations of PhK 13. The convergence of the lines indicates that these two peptides act synergistically with respect to their binding to calmodulin. In other words, both peptides are capable of binding simultaneously to a single calmodulin molecule. The point of convergence in a Dixon plot occurs at the value, $-\alpha(K_i)$, where α represents the cooperativity factor between the two inhibitors (29). A value of 1 for α indicates no cooperativity, values of $\alpha > 1$ indicate negative cooperativity, and values of $\alpha < 1$ indicate positive cooperativity. The point of convergence in Fig. 5*A* occurs at approximately 6 nM, which is comparable to the K_i value estimated for PhK 13 (6 nM; Fig. 2) thus indicating $\alpha \approx 1$. Similarly, the point of convergence of lines in Fig. 5*B* is approximately 20 nM, which also indicates $\alpha \approx 1$ since the K_i value for PhK 5 was estimated to be 20 nM (Fig. 2). When the data in Fig. 5 were fit to Equation 2 using a weighted, nonlinear least squares procedure (as described under "Experimental Procedures"), α was estimated to have a value of 0.6, indicating the possibility of modest positive cooperativity between the two peptides.

Results from several of the experiments described above indicate that peptides from both of the calmodulin-binding domains of the γ-subunit competitively inhibit calmodulin binding to myosin light chain kinase (Fig. 2 and 3) and, conversely, that the calmodulin-binding domain of myosin light chain kinase (MLCK-5) competitively inhibits calmodulin interactions with the γ-subunit (Fig. 3). To determine whether the calmodulin-binding domain peptides derived from these two target proteins are capable of synergistically binding to calmodulin, assays were performed using mixtures of MLCK-5 and each of the two γ-subunit calmodulin-binding domain peptides (PhK 5 and PhK 13). Fig. 6*A* shows a Dixon plot of v_0/v_i as a function of the concentration of PhK 13 at different fixed concentrations of MLCK-5. Fig. 6*B* shows the same type of plot, but with PhK 5 and MLCK-5. In both plots, the lines appear to be parallel to one another indicating the lack of synergistic binding between either of the PhK peptides and MLCK-5. These data indicate that the binding to calmodulin of MLCK-5 and either of the PhK calmodulin-binding peptides is mutually exclusive.

DISCUSSION

The results presented here indicate that there are two distinct, nonoverlapping, high affinity calmodulin-binding domains in the C-terminal sequence of the γ-subunit of phosphorylase kinase. The binding of both of the domains to calmodulin is Ca^{2+}-dependent. The synergism between PhK 13 (a peptide from domain N) and PhK 5 (from domain-C) in binding calmodulin suggests that in the intact polypeptide

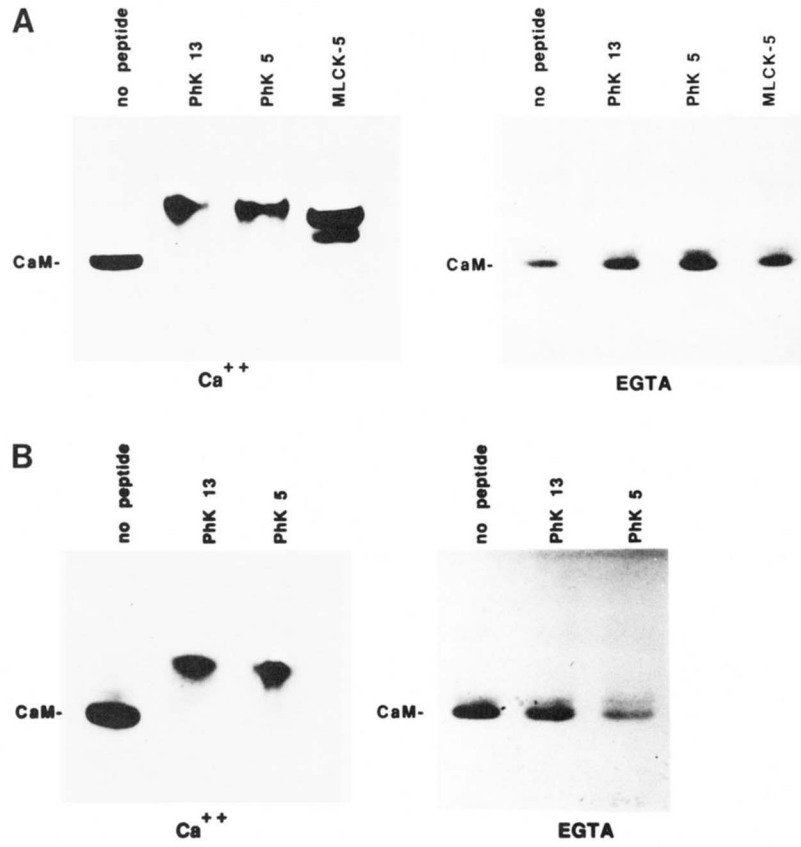

FIG. 4. **Electrophoretic mobility shift assay of calmodulin in the presence of PhK 5 and PhK 13 peptides.** *A*, calmodulin (120 pmol) was incubated with the indicated peptides (240 pmol) in the presence of 4 M urea, 100 mM Tris, pH 8.0, and 1 mM Ca^{2+} (*left panel*) or 5 mM EGTA (*right panel*) in a total volume of 20 μl for 10 min. The resultant mixtures were analyzed by changes in calmodulin electrophoretic mobility on 10% polyacrylamide gels in the presence of urea as described in Ref. 24. *B*, the electrophoretic mobility of calmodulin was determined under nondenaturing conditions. Incubations were the same as described for *A* except that no urea was included and the gel contained 7.5% glycerol.

these two domains are probably oriented such that both can simultaneously and optimally interact with calmodulin. Thus, our results suggest that extensive noncontiguous regions of the γ-subunit are involved in binding calmodulin. Multiple interactions between the γ-subunit and calmodulin (δ-subunit) involving distinct regions on both proteins may explain why strongly denaturing conditions as well as chelators are needed to dissociate the two subunits (5, 8). In other words, because of the synergism between the two domains, the γ-subunit polypeptide may have an extremely high affinity for calmodulin in the absence of Ca^{2+}, even though each individual domain binds calmodulin in a Ca^{2+}-dependent manner. Both domains of the γ-subunit appear to be involved in γ-subunit reactivation, a refolding process that uses Ca^{2+}/calmodulin as a template, since peptides from both domains can inhibit this process. This suggests that proper binding and orientation of both domains with respect to calmodulin is necessary for successful refolding of the γ-subunit.

The nominal boundaries of the two calmodulin-binding domains of the γ-subunit of phosphorylase kinase are residues 287–331 for domain-N and 332–371 for domain-C. These boundaries include the sequences of all the inhibitory peptides in each of the respective domains. It is likely that the minimal essential sequences of each domain are contained in much shorter segments. The primary structure of domain-N and domain-C do not share any sequence homology, nor does the predicted secondary structure of the two domains (not shown) show any significant similarity. The region encompassing residues 341–361 of the γ-subunit was previously identified by DeGrado *et al.* (31) as a putative calmodulin-binding domain of the γ-subunit. A computer algorithm was used that looked for sequences with large hydrophobic moments and net basic character, since the ability to form a basic amphipathic helical structure is believed to be a common structural feature in many target protein calmodulin-binding domains. The sequence identified by DeGrado and co-workers (31) is contained within PhK 5 (342–366), the most inhibitory peptide in domain-C (K_i = 20 nM). A synthetic peptide corresponding to residues 341–361 was prepared by these investigators and was shown to bind calmodulin with high affinity (estimated K_d = 11 nM; Ref. 31). The high affinity of this peptide suggests that this sequence probably includes all of the determinants that are necessary and sufficient for the binding of domain-C to calmodulin. It is interesting to note that domain-N was not predicted to be a likely calmodulin-binding domain by DeGrado's algorithm, evidently because this domain lacks the typical structural features. The sequence spanning domain-N has a propensity for forming a β-turn followed by a β-strand structure according to Chou-Fasman (32) probabilities (analysis not shown). The high affinity of PhK 13 (K_i = 6.5 nM) in domain-N thus suggests that an amphipathic helical structure may not be an obligatory feature for calmodulin binding. Studies are currently in progress to identify the secondary structural properties of PhK 13. Lukas *et al.* (33) identified the region spanning residues 322–345 as the calmodulin-binding domain of the γ-subunit and reported that the corresponding synthetic peptide binds calmodulin with a K_d value of 20 nM. This sequence is contained entirely within PhK 9 (residues 322–346; see Fig. 1). However, as indicated in Figs. 2 and 3, PhK 9 did not inhibit either MLCK activation or γ-subunit reactivation. PhK 9 also did not shift the electrophoretic mobility of calmodulin in a mobility shift assay similar to those shown in Fig. 4 (data not shown). At present, we have no explanation for these conflicting observations.

The sequences of skeletal muscle isoforms of the γ-subunit

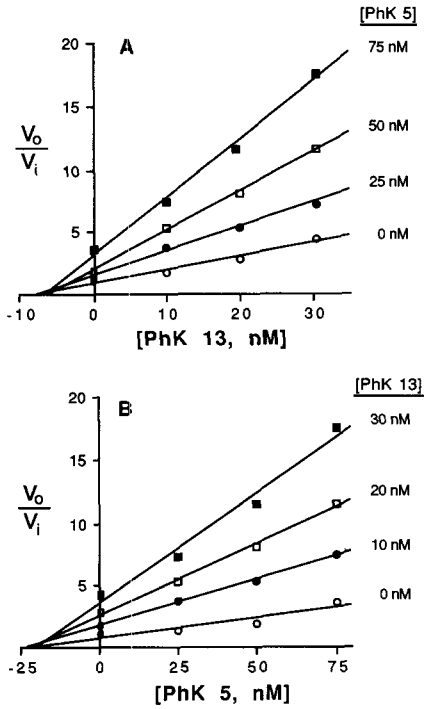

FIG. 5. **Inhibition of myosin light chain kinase activity in the presence of a mixture of PhK 5 and PhK 13 peptides.** Data are represented as Dixon plots of: v_0/v_i *versus* PhK 13 concentration at different fixed concentrations of PhK 5 (*A*) and v_0/v_i as a function of PhK 5 concentration at different fixed concentrations of PhK 13 (*B*). The reaction rate in the presence of 10 nM calmodulin and in the absence of any inhibitor is v_0, and the reaction rate in the presence of peptide inhibitors is v_i. The experimental details are described under "Experimental Procedures."

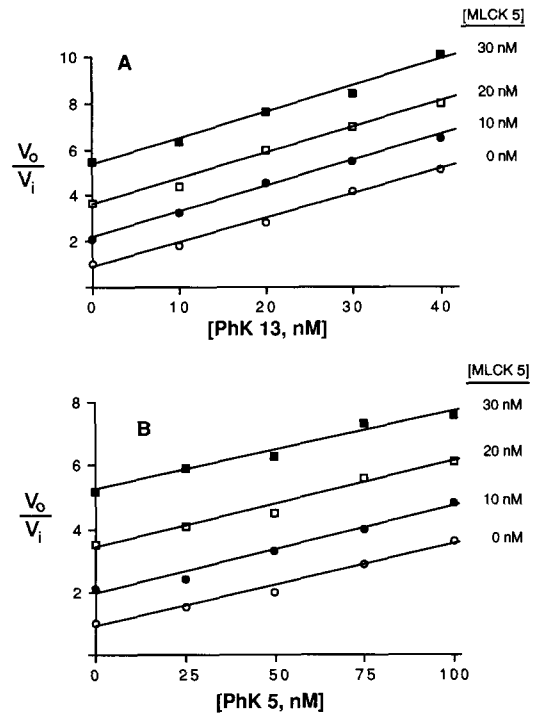

FIG. 6. **Inhibition of myosin light chain kinase activity in the presence of a mixture of MLCK-5 and either PhK 13 or PhK 5.** Shown are Dixon plots of: v_0/v_i *versus* PhK 13 concentration at different fixed concentrations of MLCK-5 (*A*) and v_0/v_i *versus* PhK 5 concentration at different fixed concentrations of MLCK-5 (*B*). The reaction rate in the presence of 10 nM calmodulin and in the absence of any inhibitor is indicated as v_0, and the reaction rate in the presence of inhibitor peptides is denoted as v_i. The experimental details are described under "Experimental Procedures."

from rat, mouse, and rabbit are highly homologous, showing 93% or greater identity to one another (11–14). A putative protein serine kinase related to the skeletal muscle γ-subunit has recently been identified by Hanks in HeLa cell extracts using homology probing (15, 34). The sequence of this protein shows 72% homology with the γ-subunit of skeletal muscle phosphorylase kinase. Northern blot analysis indicates that the HeLa cell isoform of the γ-subunit is especially enriched in rat testis (15), but is not found in rat skeletal or cardiac muscle. Enzymatic characterization of this isoform of γ-subunit has not yet been done. The sequence corresponding to calmodulin-binding domain-C in this non-muscle isoform of γ-subunit shows significant similarity with the skeletal muscle isoform (Fig. 7). This observation is consistent with this region having an important functional role, such as binding calmodulin. The sequence corresponding to domain-N is not as highly conserved in the two isoforms as is domain-C. There are at least two possible explanations for this: (i) despite the sequence dissimilarities between the two domain-N regions, all of the essential determinants for binding the δ-subunit are nevertheless present in both isoforms; (ii) the non-muscle isozyme has a somewhat different form of Ca^{2+}-dependent regulation and/or subunit interaction than the skeletal muscle isozyme. It is interesting to note that domain-N, which lacks a tryptophanyl residue in the skeletal muscle isoform, contains a tryptophanyl in the corresponding sequence of the non-muscle isozyme. Tryptophanyl residues are present in many of the calmodulin-binding domains that have been identified to date, and it has been suggested that this residue may play an important role in calmodulin-binding (35, 36). Although a tryptophanyl is present in domain-C, it does not appear to be a critical determinant for calmodulin interaction in domain-N.

Both of the calmodulin-binding domains of the γ-subunit of phosphorylase kinase show interesting similarities with specific regions of sequence in various forms of troponin I (TnI), the inhibitory subunit of troponin (Fig. 7). This is not too surprising considering the number of structural and functional similarities between phosphorylase kinase and troponin, in particular, that calmodulin and troponin C (TnC) are homologous proteins (reviewed in Ref. 37), which function as integral Ca^{2+}-regulatory subunits, and which do not readily dissociate from their respective complexes, even in the absence of Ca^{2+} (5, 6, 38). The regions of greatest sequence similarity between the γ-subunit and the different TnIs are the same regions that are the most highly conserved between different forms of TnI (Fig. 7). These regions also correspond to the PhK peptides with the highest affinity for calmodulin (Fig. 2). Domain-N of the γ-subunit shows sequence similarity (in particular the sequence, Arg-Gly-Lys-Phe-Lys) with a region in the TnIs that is known to be important for its binding to both TnC and actin (39–41). This sequence similarity was previously noted by Buschmeier *et al.* (42). The predicted secondary structures in this region are also similar, with domain-N (data not shown) and the corresponding segment of the TnIs (43, 44) both showing a propensity to form a β-turn followed by a short β-strand structure. The sequence of domain-C of the γ-subunit is also similar to a segment of TnI that is highly conserved in all forms of TnI (Fig. 7), with the sequence Val-Lys-Lys being identical in the γ-subunit and the different TnIs. Both domain-C (data not shown) and the corresponding region in the TnIs (43, 44) are predicted to

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A  [290-374] FERCEGSQPWNLTPRQRFRVAVWTVLAAGRVALSTHRVRPLTKNALLRDPYALRSVRHLIDNCAFRLYGHWVKKGEQQNRAALFQ
             *           :** :*:*   ****  *:    :**:*:*:  ::******* :*:***  ***:******** ********

B  [287-371] FQQYVVEEVRHFSPRGKFKVICLTVLASVRIYYQYRRVKPVTREIVIRDPYALRPLRRLIDAYAFRIYGHWVKKGQQQNRAALFE
                          DOMAIN-N                                  DOMAIN-C

C  [117-185] ITEIADLTQKIFDLRGKFKRPTLRLRVRISADAMMQALLGTxxxxxxxxxxxxxxxxxRAKETLDLRAHLKQVKKEDTEKENREVG
                    : *  *****   * :   :       :                      *  :    *   : ***

D  [123-186] DYEINELNIQVNDLRGKFIkPTLk-KVSKYENKFAKL---Qxxxxxxxxxxxxxxxxx KKAAEFNFRNQLKTVKKKEFELE-DDKG
                   :     ****    *   *       :                        :    : **   : ***

E  [  89-156] SKELEDMNQKLFDLRGKFKRPPLR-RVRMSADAMLKALLGSxxxxxxxxxxxxxxxxxKHKVCMDLRANLKQVKKEDTEKE-RDVG
                    : *  *****   *          : :                       :       *   : ***
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FIG. 7. **Comparison of the primary structures of the calmodulin-binding domains of the γ-subunit of phosphorylase kinase and the C-terminal sequences of various isoforms of TnI.** Sequences of a non-muscle isoform of γ-subunit (15) (*A*), rabbit cardiac muscle TnI (50) (*C*), crayfish tail muscle TnI (44) (*D*), and rabbit fast skeletal muscle TnI (51) (*E*) are compared to the sequence of the γ-subunit of rabbit skeletal muscle phosphorylase kinase (2) (*B*). Alignments were performed by eye, except for alignments between various TnI sequences which are from Kobayashi *et al.* (44). A 16-residue gap (*xxxx*) was inserted in all TnI sequences to obtain maximal alignment with the γ-subunit sequence. Amino acid identities (*) and conservative substitutions (:) with respect to the skeletal muscle γ-subunit sequence are indicated *below* each sequence. The sequences of PhK 13 and PhK 5, the highest affinity calmodulin-binding peptides in domain-N and domain-C, respectively, are *underlined* in the skeletal muscle γ-subunit sequence. Amino acid residues are represented as standard *one-letter code*, except for the 2 trimethyllysyl residues in the crayfish TnI sequence which are indicated as *k*. The nominal boundaries of phosphorylase kinase calmodulin-binding domain-N and domain-C (see "Discussion") are enclosed in *boxes* and *labeled* as such.

form an α-helical structure. The function of this sequence in TnI is not presently known, but the similarity to a corresponding region in the γ-subunit with calmodulin-binding function suggests a possible role in binding TnC. It is interesting to note that there are between 16 and 20 additional residues in the γ-subunit compared to the TnIs that separate the two regions of similarity described above. The presence of this additional structure is the most likely reason that the overall similarity between the γ-subunit and the TnIs has not been previously noted. It is difficult to know exactly where the extra residues lie in relation to the TnI sequences because of the low level of similarity in this region; however, it is possible that some of the "extra" residues found in the γ-subunit sequence may have important subunit interaction functions. For instance, the segment spanning residues 342–346, which lies in the region of "extra" structure, appears to be necessary for interactions with calmodulin since PhK peptides lacking these residues (*e.g.* PhK 4) have significantly lower affinity than those PhK peptides that contain them (*e.g.* PhK 5 and PhK 6).

In order to determine the possible evolutionarily relatedness of the γ-subunit and TnI, the C-terminal sequences from various forms of TnI were compared with the C-terminal sequence of rabbit skeletal muscle γ-subunit (residues 290–374) using the PCOMPARE program (Dayhoff mutation data matrix-78; Ref. 45) from the IntelliGenetics PC/Gene sequence analysis package. For each sequence comparison the following values were used in the program: bias parameter = 60; gap penalty = 40; number of random runs = 50. The gap penalty was reduced from the recommended default value of 60 to minimize the effect of the extra residues present in the γ-subunit between the regions of high similarity. The alignment scores obtained were 2.8, 1.5, and 1.9 for rabbit cardiac, crayfish tail, and rabbit fast skeletal muscle TnI, respectively, indicating that although the sequences are similar, the TnI sequences and the γ-subunit sequence could well have arisen by chance. This suggests a convergent form of evolution for these two functionally similar proteins, although it is also possible that both proteins are derived from an ancestral target sequence (or sequences) that has diverged during evolution to give the specialized features observed in each of the contemporary proteins. Characterization and comparison of the genes for these proteins may shed some light on the relationship of their evolutionary histories.

Although most calmodulin target proteins appear to possess a single calmodulin-binding domain contained within a relatively restricted region of the molecule (46), the presence of two calmodulin-binding domains in the same polypeptide chain has been previously suggested in the case of adenylate cyclase from *Bordetella pertussis* (47) and in rabbit skeletal muscle phosphofructokinase (42). The *Bordetella* cyclase is unusual in that it binds and is activated by calmodulin in the presence and absence of Ca^{2+}. The two domains in this enzyme thus appear to be more involved in recognizing and binding calmodulin than in mediating Ca^{2+}-dependent enzyme regulation. Muscle phosphofructokinase, on the other hand, is able to bind two molecules of calmodulin per polypeptide chain in a Ca^{2+}-dependent manner and, therefore, requires two separate calmodulin-binding domains for this capability. In contrast to the aforementioned enzymes, which do not appear to closely resemble the γ-subunit with respect to its interactions with calmodulin, a form of cyclic nucleotide phosphodiesterase from bovine lung has been reported that contains calmodulin as an integral subunit (48). It is likely that the catalytic subunit of this enzyme will be found to contain multiple calmodulin-binding domains that act in concert to regulate enzymatic activity.

It has previously been shown that synthetic peptides based on the calmodulin-binding domain of myosin light chain kinase do not affect the Ca^{2+}-dependent catalytic activity of nonactivated phosphorylase kinase (49), an activity which is regulated by the δ-subunit interacting directly with the γ-subunit. The inability of myosin light chain kinase peptides to interfere with δ-subunit function in the holoenzyme complex indicates that either the myosin light chain kinase peptides are unable to interact with the δ-subunit when it is bound to the γ-subunit, or that the myosin light chain kinase peptides do not bind to the same sites on the δ-subunit as the γ-subunit and thus do not inhibit their interactions. This latter possibility is consistent with the conclusions of Cohen *et al.* (5) which are based on the observation that purified phosphorylase kinase can substitute for calmodulin in activating target enzymes such as myosin light chain kinase. In the present study, a synthetic myosin light chain kinase

peptide was shown to inhibit the calmodulin-dependent reactivation of denatured γ-subunit (Fig. 3*B*). Moreover, peptides from each of the two γ-subunit calmodulin-binding domains inhibited myosin light chain kinase activity (Figs. 2 and 3*A*) and competitively inhibited myosin light chain kinase peptide binding to calmodulin (Fig. 6, *A* and *B*). These observations indicate that the δ-subunit of phosphorylase kinase is not capable of simultaneously binding or activating target enzymes such as myosin light chain kinase when bound to the γ-subunit in the holoenzyme complex. It is possible that the observations of Cohen *et al.* (5) can be attributed to a small amount of exogenous calmodulin (δ′-subunit) contaminating the preparation of phosphorylase kinase or to the release of a small quantity of δ-subunit from denatured or proteolyzed holoenzyme.

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